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SUBCUTANEOUS VS. INTRAMUSCULAR ADMINISTRATION OF PENICILLIN

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FOR the parenteral administration of penicillin, the intramuscular route has been recommended as the most practical one when intermittent injections are given.[1] Intravenous injections, though not so painful, are less desirable because they do not produce a sufficiently sustained plasma concentration of penicillin. On the other hand, subcutaneous injections, which should at least theoretically give more sustained plasma levels than either intramuscular or intravenous injections, have been viewed with disfavor because of the irritation produced. Accordingly, the most recent directive of the War Production Board[2] has stated that the subcutaneous route should be avoided.

These recommendations have been based on the properties of the penicillin commercially available at the time. That the degree of purity of the penicillin is the important factor in determining the intensity and duration of the pain produced by the injections has been shown by Herwick and co-workers.[3] Now that pure or almost pure preparations of penicillin have been made available for clinical use, the question of the most desirable route for parenteral administration should be re-examined. The results of such an investigation are summarized in this report.

METHODS

The following penicillin preparations were used for this study:

1. An almost pure amorphous sodium penicillin (1,550 units per milligram) put up in 200,000 unit vials. This will be spoken of as high potency sodium penicillin (amorphous).

2. Crystalline sodium penicillin (1,400 units per milligram) in 200,000 unit vials.

3. Pure crystalline potassium penicillin (1,631 units per milligram) in 100,000 unit vials. (An earlier amorphous preparation used in a few instances contained 1,501 units per milligram.)*

The penicillin was dissolved in sterile physiologic saline solution in a concentration of 10,000 units per cubic centimeter. When injected intramuscularly, the triceps or deltoid muscle was utilized, a 1½ inch, 22 gauge needle being

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employed. For subcutaneous injections, the upper arms or thighs were chosen, and the regular ½ inch, 26 gauge hypodermic needles were employed.

The penicillin preparations were injected subcutaneously or intramuscularly in doses of 10,000, 15,000, or 20,000 units into hospital control subjects who were young or middle-aged patients, not acutely ill, and who had no demonstrable circulatory or renal disturbances. Plasma penicillin concentrations were determined at one-half, two and one-half, and three hours after the injections by the serial dilution *Bacillus subtilis* method of Randall, Price, and Welch[4] as modified by Hickey.[5] In the majority of injections of penicillin, the subcutaneous and intramuscular comparisons were made in the same patient; however, since there was a great degree of variability of the plasma levels achieved with any penicillin injection even in the same patient by the same route, the results have been evaluated by the determination of the average of plasma values for any type of injection.

In order to determine whether human blood, even in the absence of penicillin, has inhibitors of growth of *B. subtilis*, as has been alleged,[6] control blood samples were taken for penicillin estimation before penicillin was administered in forty assays. In addition, blood specimens from eighty hospital patients not on penicillin or sulfonamide therapy were analyzed for penicillin. In the total of 120 plasma specimens, only one showed a level equivalent to 0.03 unit; the remainder gave 0 levels. These findings, supplemented by the many 0 levels in the hundreds of assays made during these and other studies, have convinced us that, at least with our use of the method and possibly because of the employment of citrated plasma, there is little danger of finding positive penicillin levels in plasma specimens that do not contain penicillin.

RESULTS

Pain.—Preliminary experiments have already demonstrated that with none of the three preparations was pain an important factor for either the subcutaneous or the intramuscular route. When any of these three preparations was given alternately for therapeutic purposes with the common impure commercial preparations (six different brands commonly purchased by the hospital), the patient had no difficulty distinguishing between the relatively pure and the low potency brands, even though they did not see the solutions injected. The purified material produced only the pain of injection; there was no prolonged pain. However, the impure yellow preparations gave a "bee-sting" type of irritation that lasted from three to fifteen minutes. This finding was confirmed by my personal experience. Similarly, in all the assays the subjects did not complain of any prolonged pain following either subcutaneous or intramuscular injection. In fact, the subcutaneous injections, made as they were with a small needle, were for the most part regarded as less annoying. It is particularly worth noting that the potassium penicillin, which previously had never been used in any extensive series of injections, produced no more pain or other reactions than did the two sodium preparations. Also, in this series of 206 assays, there were no recognized cases of urticaria or other allergic manifestation, which finding may be related to the purity of the preparation used.

TABLE I. FREQUENCY DISTRIBUTIONS OF PLASMA PENICILLIN LEVELS AFTER SUBCUTANEOUS OR INTRAMUSCULAR INJECTIONS OF PURIFIED PENICILLIN PREPARATIONS

DOSE (UNITS)	PLASMA PENICILLIN CONCENTRATION (UNITS PER C.C.)	HIGH POTENCY SODIUM PENICILLIN (AMORPHOUS)						POTASSIUM PENICILLIN						CRYSTALLINE SODIUM PENICILLIN					
		SUBCUTANEOUS HOUR			INTRAMUSCULAR HOUR			SUBCUTANEOUS HOUR			INTRAMUSCULAR HOUR			SUBCUTANEOUS HOUR			INTRAMUSCULAR HOUR		
		½	2½	3	½	2½	3	½	2½	3	½	2½	3	½	2½	3	½	2½	3
10,000	1.0																		
	0.5	3			3			1			1			1			1		
	0.25	8			6			8			8			7			7		
	0.125	1	1		3			1			1	1		2			2		
	0.062		3			7	2		1			3	2					1	1
	0.031		6	4		2	2		9	6		6	5		8	7		7	5
	0		2	8		3	8			4			3		2	3		2	4
15,000	1.0																		
	0.5	3			1			5			5			4			3		
	0.25	6			6			3			5	1		13			6		
	0.125	1			3	1			2			1		1			1		
	0.06		3	2		1	3	3	3	1		5	3		6	3		4	2
	0.03		5	4		6	3		4	4		3	3		8	10		5	5
	0		2	3		2	4		2	6			4		4	5		1	3
20,000	1.0	2			1			1			3			3			1		
	0.5	8			12			6			3			5			8		
	0.25	5	1		2			3			4			2	1		3		
	0.125		6	1		7						1				1		2	
	0.062		5	7		5	2		6	2		5	2		6	1		8	5
	0.031		3	5		2	8		3	6		4	6		2	6		2	5
	0			2		1	5		1	2			2		1	2			2

Comparison of Plasma Penicillin Levels.—The frequency distribution of plasma Penicillin levels at one-half hour, two and one-half hours, and three hours after subcutaneous or intramuscular injections of 10,000, 15,000, or 20,000 units of penicillin is shown in Table I. A total of 206 assays were made involving 618 determinations of plasma penicillin concentrations. It will be seen that there was a considerable variation in the levels obtained for any one type of injection in the several subjects but that usually the modal level could be easily determined. In general, the plasma level for 10,000 units by either subcutaneous or intramuscular injections at one-half hour was 0.25 unit per cubic centimeter; at two and one-half hours, 0.03 unit per cubic centimeter; and at three hours, 0 unit per cubic centimeter (that is less than 0.03). For 15,000 units, there was no great difference at one-half hour or at two and one-half hours, but at three hours the prevailing level was 0.03 unit per cubic centimeter. For 20,000 units, the modal value was 0.50 unit per cubic centimeter at one-half hour, 0.06 unit per cubic centimeter at two and one-half hours, and 0.03 unit per cubic centimeter at three hours.

For a more rigid comparison of the levels obtainable after the subcutaneous and intramuscular injections of the different dosages of the different preparations, the mean levels were tabulated in Table II. It should be recognized that the use of the arithmetic mean in averaging values that progress geometrically is likely to give too much weight to isolated high values and that it might be better to obtain the average by giving an arithmetically progressing weighting to each level or otherwise to use the geometrical mean.

TABLE II. AVERAGE PLASMA PENICILLIN LEVELS AFTER SUBCUTANEOUS OR INTRAMUSCULAR INJECTION OF PURIFIED PENICILLIN PREPARATIONS

DOSE (UNITS)	TYPE OF INJECTION	HIGH POTENCY SODIUM (AMORPHOUS)			CRYSTALLINE SODIUM			CRYSTALLINE POTASSIUM			TOTAL AVERAGE		
		½ HR.	2½ HR.	3 HR.	½ HR.	2½ HR.	3 HR.	½ HR.	2½ HR.	3 HR.	½ HR.	2½ HR.	3 HR.
10,000	Subcutaneous	.302	.042	.010	.263	.034	.019	.250	.025	.022	.272	.034	.017
	Intramuscular	.281	.042	.015	.263	.050	.028	.250	.029	.022	.265	.040	.022
15,000	Subcutaneous	.313	.035	.025	.312	.056	.016	.300	.035	.028	.308	.042	.023
	Intramuscular	.238	.037	.028	.375	.078	.028	.313	.041	.028	.308	.052	.028
20,000	Subcutaneous	.483	.094	.048	.475	.047	.031	.600	.069	.038	.519	.070	.039
	Intramuscular	.500	.065	.025	.550	.056	.031	.480	.068	.047	.510	.063	.034

Nevertheless, in spite of the handicap of the method, it could readily be seen that there was no appreciable difference between the levels obtained after subcutaneous injections and those after intramuscular injections. It could also be recognized that all three preparations, the two sodium penicillins and the potassium, gave essentially the same results. The average levels for each interval for all the injections (see Table II) agree fairly closely with the modal values previously mentioned. The plasma levels found indicate that, even with such a low dosage as 10,000 units of these purified preparations every three hours, there is likely to be therapeutic levels during at least two and one-half hours and that the chief value of giving 15,000 or 20,000 units is to assure a therapeutic level in the last one-half hour or hour. A dosage of 15,000 units of purified penicillin every three hours would probably be effective in all infections by bacteria relatively sensitive to penicillin, which include the gonococcus, pneumococcus, *Staphylococcus aureus,* and meningococcus. On the other hand, in cases of infections by more resistant organisms, such as the *Staphylococcus albus* and *Streptococcus viridans,* where plasma levels of the order of 0.5 unit per cubic centimeter or higher may be required, dosages of 20,000 units every three hours are not high enough to insure therapeutic levels.

That subcutaneous injections of purified penicillin gave no more prolonged levels than did intramuscular injections may be due to the relative insensitivity of the serial dilution method used for the assay of penicillin. Nevertheless, producing, as it does, at least as good and prolonged levels as intramuscular injections, the subcutaneous route becomes the method of parenteral administration of choice.

SUMMARY

Comparison of subcutaneous and intramuscular injections of three purified preparations of penicillin were made in 206 assays involving 618 plasma penicillin level estimations.

There was no afterpain with either type of injection. Subcutaneous injections, because they permitted the use of a small needle, were less annoying than those given intramuscularly.

There were no essential differences between the plasma penicillin levels obtained after subcutaneous injections and those following intramuscular ad-

ministration. Also, all three purified preparations, the amorphous and the crystalline sodium penicillin and the crystalline potassium penicillin, gave essentially the same results.

There were no instances of urticaria or other forms of sensitivity in all 206 assays.

The subcutaneous route has been found from our studies to be the one of choice for the parenteral administration of purified penicillin preparations.

The author wishes to acknowledge his indebtedness to Mrs. Ellen Zimmermann, Mrs. Jeanne Hildebrandt, and Miss Margaret Casella, for their assistance in the assays.

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PERMEABILITY ALTERATIONS IN DISEASE

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IN THE course of recent investigations into the fluid and ionic balance in simian malaria,[1] a large number of determinations of "available fluid" (extracellular) volumes were done by the sodium thiocyanate method[2] (1) in normal monkeys, (2) in monkeys with fatal *Plasmodium knowlesi* infections, and (3) in monkeys with malaria which were treated subsequently with sulfadiazine and survived. In twenty-three monkeys control thiocyanate volumes ranged from 16.6 to 28.8 per cent of the body weight with a mean value of 20.8 per cent. The spread in these values is less if measurements obtained in the summer or winter are considered separately, for the monkey exhibits a larger extracellular volume in summer. There appears to be a similar seasonal variation in blood, plasma, and extracellular volumes in man.[3]

Following the induction of *P. knowlesi* infection in monkeys the volume of fluid available for dilution of NaSCN increased in a progressive manner throughout the course of the disease. This progressive change in two animals (5AS and 6AS) and the return toward normal following chemotherapeutic intervention is shown in Fig. 1. At first it was thought that water shifts were occurring and that the extracellular compartment was becoming superhydrated. However, as the parasitemic level rose, the *apparent* extracellular fluid volume increased to such a degree that it became obvious that the changes could not be due to water shifts alone. In all cases of malaria fatalities in which measurements were made within twenty-four hours preceding death, the volume of fluid available for dilution of thiocyanate increased until it exceeded 50 per cent of the body weight. A few animals in which determinations were made within the six-hour period preceding death revealed available fluid volumes of 64 to 66 per cent of the body weight. Furthermore, these volume changes occurred over a period of time in which plasma volumes fell slightly and the animals lost weight.

Harrison, Darrow, and Yannet,[4] employing the desiccation technique, found that the total body water of the monkey is approximately 65 per cent of the body weight. It is obvious at once that NaSCN determinations do not, in the malarious animal, represent the extracellular volume.

At autopsy, neither the peritoneal cavity nor the intestine exhibited any gross accummulation of fluid. Cut surfaces of all organs appeared dry and dehydrated. Further, the time-concentration curves of NaSCN in plasma were of no steeper slope than in normal animals, indicating that NaSCN was neither being destroyed nor lost from the body at an increased rate.

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In five animals which died of malaria, tissue thiocyanate determinations were made immediately after death on heart, liver, spleen, and muscle. The amount of thiocyanate found in these tissues varied, liver having the highest and muscle the least (Table I). However, in all tissues, the amount found was larger than could have been resident in the chloride space of the tissue. In the course of simian malaria, at least, the dilution of sodium thiocyanate is no longer a measure of the extracellular fluid. The conclusion is inescapable that, in malaria, sodium thiocyanate enters the cells and becomes diluted by the intracellular water.

TABLE I. RESULTS OF TISSUE AND PLASMA THIOCYANATE DETERMINATIONS

ANIMAL	TISSUE	SCN IN Cl SPACE OF 1,000 GM. TISSUE* (MG.)	SCN FOUND IN 1,000 GM. TISSUE (MG.)	SCN IN 1,000 C.C. INTRACELLULAR WATER (MG.)	SCN SPACE AS PER CENT BODY WEIGHT
9AT	Muscle	4.82	5.1	0.28	28.8
11AK	Muscle	3.98	5.1	1.12	33.1
7AK	Muscle	2.98	5.0	2.01	39.8
8AK	Muscle	2.16	5.2	2.04	48.2
10AT	Muscle	1.81	5.1	3.29	54.6
9AT	Liver	13.49	16.32	2.83	28.8
11AK	Liver	11.17	22.44	11.27	33.1
7AK	Liver	8.37	15.0	6.63	39.8
8AK	Liver	6.04	15.6	9.56	48.2
10AT	Liver	5.07	17.34	12.27	54.6
9AT	Spleen	13.10	10.20	none	28.8
11AK	Spleen	10.84	18.36	7.52	33.1
7AK	Spleen	8.13	7.0	none	39.8
8AK	Spleen	5.87	8.84	2.97	48.2
10AT	Spleen	4.93	22.44	17.51	54.6
9AT	Plasma	35.41	31.90		28.8
11AK	Plasma	29.30	26.40		33.1
7AK	Plasma	21.98	19.80		39.8
8AK	Plasma	15.87	14.30		48.2
10AT	Plasma	13.32	12.00		54.6

*Calculated from the plasma level of SCN and Lepore's[8] values for the chloride space of muscle, liver, and spleen.

That such a permeability change may be a general phenomenon accompanying febrile diseases is suggested by the following findings: (1) Repeated injections of typhoid vaccine in dogs over a period of ten days effect a significant increase in *apparent* extracellular volume.[5] (2) Measurements made by Ling[6] on patients with neglected compound fractures who had developed bacteremias with concomitant temperature elevations showed increases in the volume of fluid available for the dilution of NaSCN of the same order of magnitude as is being reported here in simian malaria. (3) NaSCN volumes measured by Harrel[7] in patients with spotted fever likewise have indicated permeability alterations.

Although the survival time of Ling's patients did not allow repeated determinations of the NaSCN volume, the recovery phase of this permeability alteration has been studied in one group of the monkeys reported here and has been noted by Harrel in human patients with spotted fever. In the monkey the recovery period is long, normal permeability not being completely re-estab-

lished until about 100 days following the disappearance of clinical malaria (Fig. 1). According to Harrel, however, the NaSCN volumes of spotted fever patients subjected to therapy quickly returned to normal.

The indication that, in malaria, the permeability of cellular membranes is altered to the extent of allowing free passage of a foreign ion (SCN), which, in the normal animal, is generally excluded from the cell water, introduces a possible new interpretation of previous studies on native ion concentrations in paludic blood.

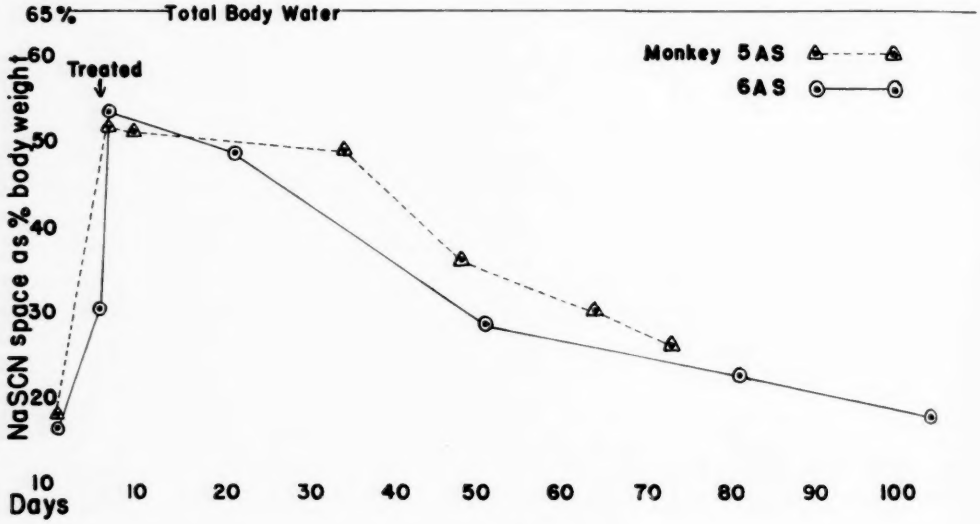

Fig. 1.

Pinelli,[9] Velick and Scudder,[10] Zwemer, Sims, and Coggeshal,[11] and Flosi[12] have all reported a decrease in blood sodium and/or an increase in blood potassium concentrations in malaria. Attempts have been made to correlate these findings with red cell destruction. Edelman and associates[13] have described similar ionic shifts occurring in the blood of patients subjected to pyrexia induced by physical means alone (hypertherm). The logical explanation of all of these results lies in the demonstration of generalized permeability alterations in the pyrexic state.

SUMMARY

1. The extracellular fluid volume as measured by the dilution of NaSCN increases in (1) simian malaria, (2) patients with spotted fever, and (3) patients with bacteremia who have concomitant temperature elevations.

2. Extracellular fluid volumes in these cases may increase to the point of being equal to the calculated total body water.

3. Tissue NaSCN determinations made immediately after death in monkeys in which *P. knowlesi* infections were fatal show larger amounts of NaSCN than could be resident in the "chloride space" of the tissue.

4. In these diseases, at least, the permeability of the tissue cells is altered, allowing the foreign ion (SCN) to become diluted by the intracellular water.

5. Animals showing such permeability changes return to normal slowly following chemotherapeutic intervention.

6. It appears that permeability alterations accompany the pyrexic state.

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GLYCOCYAMINE ELIMINATION IN PATIENTS WITH MYASTHENIA GRAVIS

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MUSCLE function has been shown to improve in patients with myasthenia gravis after infusion of amino acids.[1] Certain amino acids (glycine, arginine, sarcosine) may form glycocyamine by transamidination, and glycocyamine is methylated to creatine.[2-6] Glycocyamine is continuously eliminated in the urine of human beings.[7, 8] A disturbance in the decomposition of the mentioned amino acids, if severe, would manifest itself in changes of the amount of glycocyamine eliminated, especially if the organism is overloaded with glycine. A severe decrease in transmethylation would manifest itself as a delayed or decreased formation of creatine from glycocyamine and an increased elimination of glycocyamine, especially if the organism is overloaded with glycine.

To ascertain whether the observed effect of amino acids on muscle function in patients with myasthenia gravis was due to correction of a deficiency of amino acids involved in creatine formation, the amounts of glycocyamine eliminated in the urine of patients with myasthenia gravis before and after administration of glycine were determined.

METHOD

The glycocyamine elimination of twelve patients with myasthenia gravis and twenty control subjects was determined by a modified method of Weber.[7-10] All subjects were hospitalized and kept on a 2,000 calorie mixed diet. The control subjects had either ruptured intervertebral discs or were patients with cardiac arrhythmias but well compensated. Urine was collected in separate samples for twenty-four hours and kept in an icebox. Fifteen grams of glycine were then administered orally in one dose, and the collection of urine was continued for forty-eight hours.

All urine samples were diluted to a specific gravity of 1,010. To 5 c.c. of urine 2.5 c.c. of water, 2.5 c.c. of 0.4 N hydrochloric acid, and 500 mg. of Lloyd's reagent were added. The mixture was shaken vigorously for three minutes, then centrifuged, and the residue rinsed with water, slightly acidified with sulfuric acid. The precipitate was suspended in 8 c.c. of water, treated with 500 mg. of barium hydroxide, shaken for three minutes, and centrifuged. The precipitate was washed twice with 2 c.c. of water. The three supernatant fluids were pooled, and the barium in the filtrate was precipitated with 40 per cent sulfuric acid. The mixture was centrifuged. The supernatant fluid was shaken for three minutes with 1 Gm. of basic lead carbonate and centrifuged. This

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TABLE 1. SHORT SUMMARY OF CLINICAL STATE OF TWELVE PATIENTS WITH MYASTHENIA GRAVIS

NAME	SEX	AGE	SEVERITY OF MYASTHENIA GRAVIS*	DURATION (YEARS)	THYMECTOMY	X-RAY TREATMENT	SYMPTOMATOLOGY	NEOSTIGMINE (PROSTIGMINE BROMIDE) †	
								DOSE (MG.)	ACTIVITY ON MEDICATION
Go	F	43	4+	7	No	Yes	Bedridden, severe lid ptosis, diplopia, difficulty in chewing and swallowing, severe muscular fatigability	135 (By mouth)	Sits in chair for a few hours
Ri	F	21	4+	1	No	Yes	Bedridden, severe lid ptosis, diplopia, difficulty in chewing and swallowing, severe muscular fatigability	180 (By mouth) 5 (By injection)	Chews food, walks few steps
Ro	F	26	3+	13	No	Yes	Moderate lid ptosis, occasional diplopia, occasional difficulty in chewing, severe difficulty in gait, moderate muscular fatigability	90 (By mouth)	Walks one to two blocks
An	F	21	3+	3	No	No	Moderate lid ptosis, occasional diplopia, difficulty in breathing and swallowing, occasional difficulty in chewing, moderate muscular fatigability	150 (By mouth)	Walks, feels comfortable
Ku	M	24	2+	2	No	Yes	Moderate lid ptosis, severe diplopia, difficulty in swallowing and chewing, moderate muscular fatigability	60 (By mouth)	Walks, slight sports
Pe	F	28	1+	8	No	No	Moderate lid ptosis, moderate muscular fatigability	120 (By mouth)	Housework
Sh	M	48	1+	5	No	No	Complete lid ptosis, moderate muscular fatigability	30 (By mouth)	Works, horseback riding
Sch	M	25	1+	5	Yes	No	Slight lid ptosis, mild muscular fatigability	15 to 30 (By mouth)	Works as an engineer
Mu	F	30	1+	9	No	No	Moderate lid ptosis, rare diplopia, mild muscular fatigability	15 to 90 (By mouth)	Works as a cashier
Co	M	37	1+	1	No	No	Moderate lid ptosis, moderate diplopia, moderate difficulty in chewing and swallowing, mild muscular fatigability	45 (By mouth)	Walks, some work
Vi	F	23	1+	8	No	No	Slight lid ptosis, mild muscular fatigability	15 to 45 (By mouth)	Walks, goes to parties
La	M	48	1+	17	No	No	Severe lid ptosis	15 (By mouth)	Works as a tailor

*Most severely ill patient, 4 plus; least severely ill patient, 1 plus.
†Hoffmann-La Roche, Inc., Nutley, N. J.

supernatant fluid was shaken for eight minutes with 1 Gm. permutit and centrifuged. To 5 c.c. of the supernatant fluid, 1 c.c. of 10 per cent sodium hydroxide solution was added, and the test tubes containing the aliquots of the solutions to be analyzed were placed in an ice water bath. In this bath were also the α-naphthol (0.04 per cent), urea (40 per cent), and hypobromite (0.66 c.c. of liquid bromine in 100 c.c. of 5 per cent sodium hydroxide) solutions. After ten minutes 1 c.c. of the α-naphthol solution was added to each test tube, the contents shaken, 1 c.c. of the chilled urea solution added, and the contents shaken again. After another five-minute interval 0.5 c.c. of the chilled hypobromite solution was added, the contents mixed by vigorous shaking, and the tube replaced in the ice bath. After twenty minutes the color was determined with a Klett-Summerson photoelectric colorimeter using filter No. 54.

Material.—A short description of the clinical state of the patients with myasthenia gravis is given in Table I.

RESULTS

The amounts of glycocyamine eliminated in the urine before and after administration of glycine are given in Table II. The men used as controls elimi-

TABLE II. ELIMINATION OF GLYCOCYAMINE IN PATIENTS WITH MYASTHENIA GRAVIS AND IN CONTROL SUBJECTS

SUBJECTS	SEX	GLYCOCYAMINE ELIMINATED DURING 24 HOURS (MG.)			
		DAY BEFORE GLYCINE	GLYCINE DAY	DAY AFTER GLYCINE	DIFFERENCE
Controls					
Da	M	59.0	79.7	58.2	20.7
Sa	M	44.5	68.5	43.0	22.0
Go	M	35.7	59.5	37.4	23.8
Fa	M	53.7	81.6	54.1	27.9
We	M	59.1	85.4	58.7	26.3
Bi	M	61.6	86.0	60.3	24.4
La	M	52.3	70.7	50.9	18.4
Ve	M	47.8	67.0	50.6	19.2
No	M	45.0	71.2	47.3	26.2
Da	F	63.0	105.2	63.9	42.2
No	F	36.5	61.3	38.9	24.8
Ho	F	82.8	122.9	80.6	40.1
He	F	80.2	138.4	80.5	58.2
Bo	F	55.7	111.2	57.4	55.7
Bu	F	33.2	62.9	36.0	29.7
El	F	70.4	90.2	68.7	19.8
La	F	63.7	114.5	62.5	50.8
Mo	F	77.2	99.0	78.4	21.8
To	F	60.7	90.3	63.9	29.7
Patients					
Ku	M	34.9	57.8	33.7	22.9
Soh	M	45.1	63.5	46.8	18.4
Sh	M	55.5	79.2	53.4	23.7
Co	M	43.2	69.0	47.8	25.8
La	M	46.6	72.4	44.4	25.8
Go	F	35.5	67.1	37.3	31.6
Ro	F	72.1	138.5	70.2	66.4
Ri	F	45.3	68.5	45.8	23.2
An	F	55.4	82.2	60.5	26.8
Pe	F	81.4	111.8	80.3	30.4
Mu	F	70.9	115.5	73.0	44.6
Vi	F	65.4	99.6	61.8	34.2

nated from 35 to 61 mg. glycocyamine daily and the women used as controls from 33 to 82 mg. per day. Patients with myasthenia gravis eliminated similar amounts of glycocyamine daily. Oral administration of glycine increased the elimination of glycocyamine from 18 to about 66 mg. in both control subjects and patients with myasthenia gravis. Patients with myasthenia gravis excreted the largest amounts of glycocyamine from four to six hours after administration of glycine. This seems to be a delay as compared to the data of Borsook and associates[8] who found that the greatest amount of glycocyamine is eliminated two hours after administration of glycine. The four- to six-hour peak lays, however, well within the limits observed with the control patients used in the present experiments.

DISCUSSION

The preceding results suggest that patients with myasthenia gravis eliminate glycocyamine in amounts similar to human controls. Furthermore, glycocyamine elimination after administration of glycine is similar in amounts in patients with myasthenia gravis and in control subjects. In so far as it is possible to determine by the amount of glycocyamine eliminated, the processes of transamidination and transmethylation are not disturbed in patients with myasthenia gravis.

It is likely that glycocyamine is formed mainly in the kidney by transamidination and is methylated mainly in the liver to form creatine.[6] Striated muscle, however, also contains minute amounts of glycocyamine (between 3 to 6 mg. per 100 Gm. of fresh rat muscle).[6] Since a local increase of glycocyamine in the muscle may not be detectable by the amounts of glycocyamine eliminated in the urine and since, according to a recently presented concept,[11] most of the symptomatology of myasthenia gravis can be due to a decreased acetylcholine synthesis, it was also ascertained whether glycocyamine modifies the synthesis of acetylcholine in vitro and whether it may modify the excitability of striated muscle. Glycocyamine is without effect on these processes. Glycocyamine does not significantly modify acetylcholine synthesis (only a 10 to 15 per cent decrease could be demonstrated with concentrations as high as 1 Gm. per 100 c.c.); it does not modify the sensitivity of muscle to indirect stimulation and chemical stimuli (acetylcholine, potassium).[12] Therefore, even if glycocyamine were to be present in increased amounts in the muscle and nerve of patients with myasthenia gravis, it would not be responsible for the severe fatigability of the muscles of these patients.

SUMMARY

From the amount of glycocyamine eliminated (before and after overloading the body with glycine) there is no evidence of any defect in transamidination and transmethylation processes in patients with myasthenia gravis.

The authors wish to express their gratitude to Dr. Joseph P. Chandler, for his valuable suggestions.

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ERYTHROCYTE FRAGILITY IN ACUTE INFECTIOUS HEPATITIS

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THE high incidence of acute infectious hepatitis in the military services has afforded an excellent opportunity for the investigation of both clinical and laboratory aspects of this disease. Changes in the fragility of the erythrocytes are of interest not only from the standpoint of establishing a differential diagnosis between this disease and hemolytic icterus but also because of the important role played by the liver in the maturation of erythrocytes. It is conceivable that the diffuse liver damage in acute infectious hepatitis may be reflected in an altered resistance of erythrocytes to hemolysis by hypotonic saline solutions. Little evidence is available in the current literature on acute infectious hepatitis regarding the effects of this disease on the fragility of erythrocytes. Most reports make no mention of studies of fragility. Havens,[1] however, obtained normal values for erythrocyte fragility in forty-two patients with infectious hepatitis. McCarty[2] agreed that this value is usually normal. Finks and Blumberg[3] carried out sixty-six tests of erythrocyte fragility and concluded that "none of these tests revealed any increased hemolysis as compared with that of the control. In fact a majority of the tests revealed an increased resistance of the red blood cell to hypotonic saline solutions."

In contrast to the majority of these reports which lead one to believe that the changes in erythrocyte fragility are of little uniformity or significance, Cohen,[4] in a study of twenty-three cases of acute infectious hepatitis, found that "clinical jaundice is accompanied by increased resistance to hypotonic hemolysis" and concluded that "the increased mean resistance is a direct function of the degree of jaundice maintained with and subsiding as does the latter." Our studies have confirmed the finding that the erythrocyte fragility is consistently decreased in acute infectious hepatitis. Further studies were conducted in an attempt to determine the cause of this altered fragility.

MATERIALS AND METHOD

This work was carried out in a United States Army Hospital assigned to a Dutch Hospital Ship operating in the Southwest Pacific. The patients were American soldiers who had developed acute infectious hepatitis in the Philippine Islands and were studied during their evacuation to General Hospitals on New Guinea, a trip which took four or five days. Icteric indices and erythrocyte fragilities were determined on a total of forty-seven patients during three different evacuations, and, as the findings were similar for these three groups of patients, the data will be presented together.

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TABLE I

	PER CENT SODIUM CHLORIDE	
	RANGE	MEAN
Initial hemolysis	0.41 to 0.45	0.429
Complete hemolysis	0.31 to 0.35	0.323
Mean hemolysis	0.360 to 0.395	0.376
Hemolysis span (difference between initial and complete hemolysis)	0.08 to 0.13	0.105

Erythrocyte fragilities were determined by a method outlined by Cohen.[3] Two-tenths of a cubic centimeter of blood was suspended in 5 c.c. of each of a series of concentrations of saline solution varying from 0.18 to 0.45 per cent. After these suspensions had stood in an icebox for three hours, the initial hemolysis was read as the highest saline concentration to show evidence of hemolysis in the supernatant fluid. The value of complete hemolysis was determined by counting the unhemolyzed cells. The highest saline concentration to contain less than twenty cells per high power field was called the level of complete hemolysis. Using this method, the values shown in Table I were obtained for fifteen normal male controls.

RESULTS

The duration of the disease at the time the first laboratory tests were carried out varied from two to forty-seven days, only nine of the patients being symptomatic for over three weeks. In Fig. 1 is shown the icteric index in Curve A and the mean erythrocyte fragility in Curve B, plotted against the duration of the disease in days for the forty-seven patients. The icteric index curve rises and the erythrocyte fragility curve falls during the first week of the disease. Both values reach the peak of abnormality in the second five-day period, then gradually return to normal. The mean erythrocyte fragility does not rise to normal (0.36 per cent) until after the third week of the disease. This corresponds closely to the return to normal of the icteric index.

Represented in Fig. 2 are changes in icteric index and mean erythrocyte fragility observed in a hospital ship corpsman who was studied throughout the course of the illness. Fortunately his blood had been used as a control for an erythrocyte fragility test five days before the onset of symptoms. At that time the mean erythrocyte fragility was found to be 0.375 per cent. The results of the laboratory tests on this patient correspond closely to mean curves seen in Fig. 1.

In Fig. 3 erythrocyte fragilities are plotted against their respective icteric indices for the forty-seven patients studied. Curve A represents the initial hemolysis at different icteric index levels. This curve falls gradually, dropping below the lower limit of normal at an icteric index of 19. The level of complete hemolysis, on the other hand, falls much more abruptly, as recorded in Curve B. This relatively more rapid fall in the level of complete hemolysis, as compared to the fall in initial hemolysis, is reflected in a marked increase in fragility span, represented in Curve C.

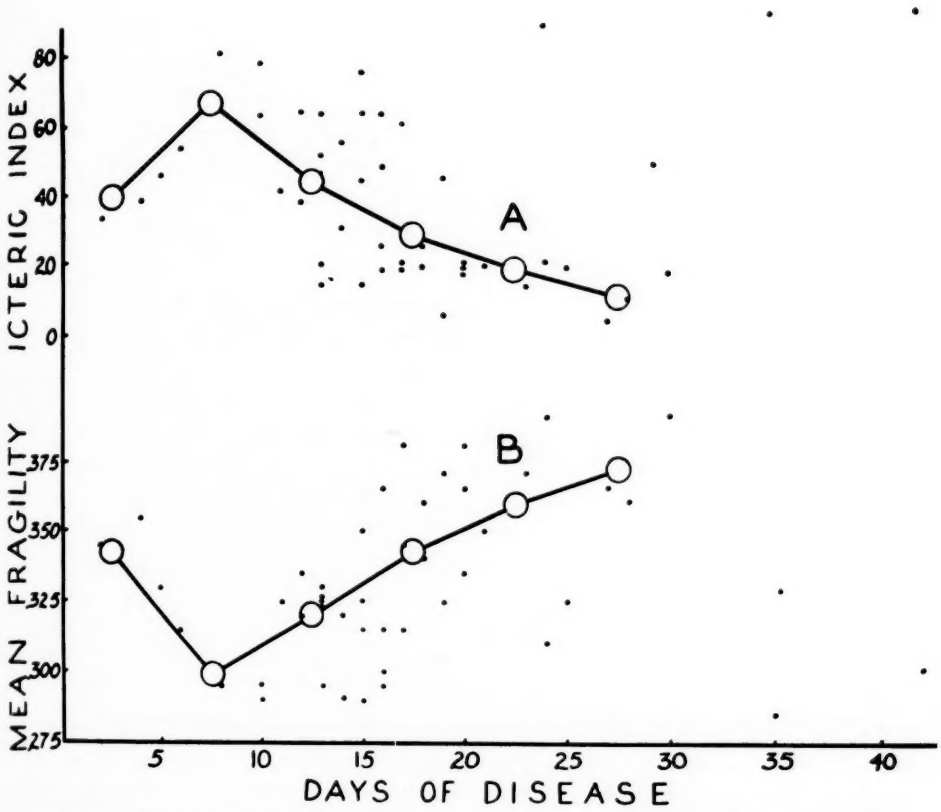

Fig. 1.—Icteric index (Curve A) and mean erythrocyte fragility (Curve B) in forty-seven patients with acute infectious hepatitis, plotted against duration of the disease in days. Curves are constructed on the mean values for each five-day period.

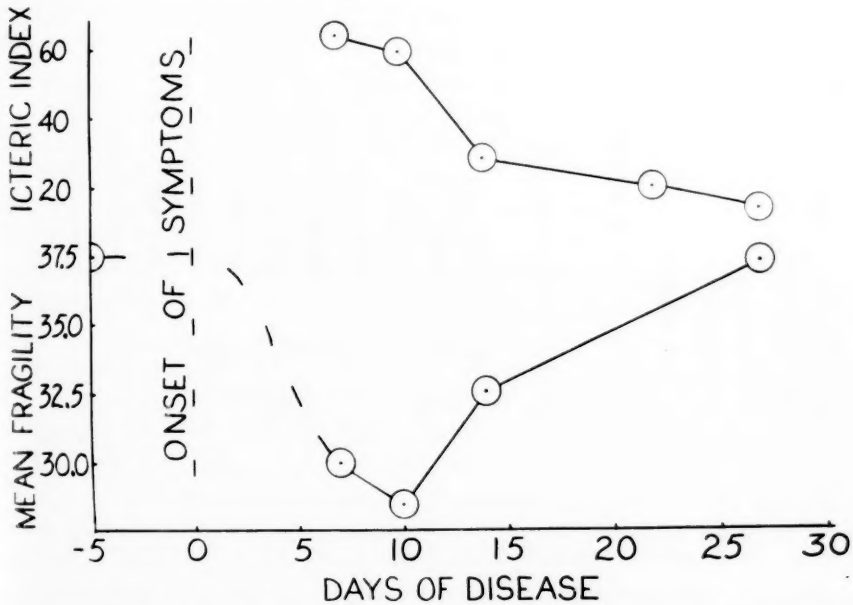

Fig. 2.—Icteric index and mean erythrocyte fragility changes in a hospital corpsman observed throughout the course of acute infectious hepatitis. His blood had been used as a control for an erythrocyte fragility study five days before the onset of symptoms.

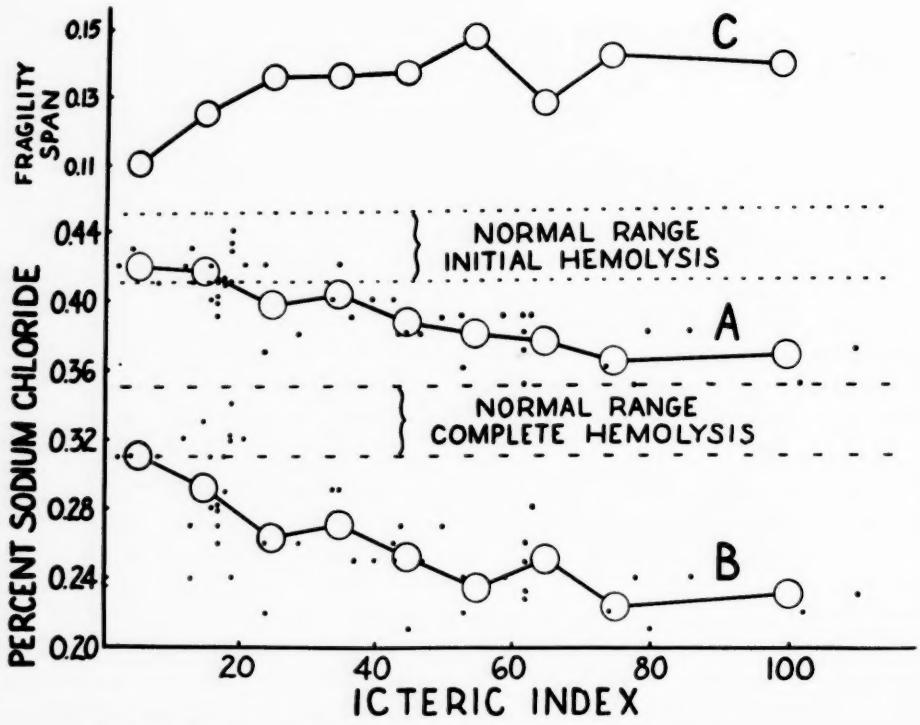

Fig. 3.—Erythrocyte fragility changes plotted against the icteric index for forty-seven patients with acute infectious hepatitis. Curve A is the initial hemolysis, Curve B is the complete hemolysis, and Curve C is the fragility span. Curves are constructed on the mean values for each 10 units of the icteric index.

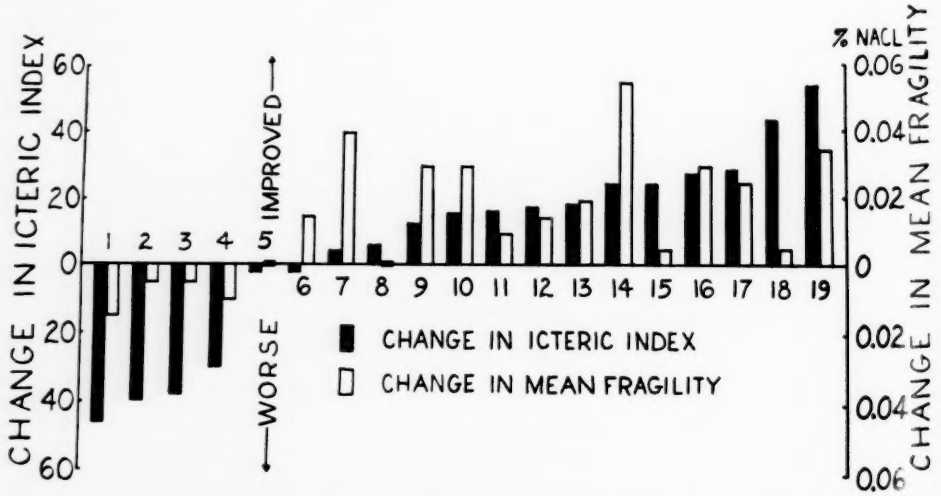

Fig. 4.—Changes in icteric index and mean erythrocyte fragility in nineteen patients with acute infectious hepatitis during a four- or five-day observation period.

DISCUSSION

The fact that erythrocyte fragility is decreased during the icteric phase of acute infectious hepatitis naturally suggests that the bile pigments of the serum may in some way increase the resistance of the cell to hemolysis by hypotonic saline solutions. This possibility has been suggested by Cohen[4] and Berk.[6]

Several observations concerning the validity of this hypothesis may be made from the data presented here. If the decreased fragility were due to an increase in bile pigment of the serum, changes in these two values in a given patient should be of proportionate magnitude. However, taking several examples from Fig. 3, marked discrepancies are found. For instance, one patient had an icteric index of 13 and complete hemolysis at 0.24 per cent, while another patient with an icteric index of 21 had erythrocytes that were completely hemolyzed within the normal range (0.32 per cent). Another striking example of the disproportionate changes in these two values is seen by comparing the patient with an icteric index of only 24 whose complete hemolysis occurred at 0.22 per cent with the patient who had an icteric index of 63 and complete hemolysis at 0.28 per cent.

Further evidence substantiating this lack of correlation is seen in the progress chart in Fig. 4. Here the magnitude of the changes in erythrocyte fragility is compared with that of the changes in the icteric index during the four- or five-day trip. "Improved" signifies a decreased icteric index and an increased erythrocyte fragility, while "worse" indicates reverse changes. Solid columns represent changes in icteric index, while hollow ones signify changes in erythrocyte fragility. The columns of the nineteen patients thus studied are arranged in the order of increasing improvement in the icteric index. In the first six patients the icteric index actually increased during the trip. The seventh patient showed a marked improvement in erythrocyte fragility and only minimal improvement in icteric index. The reverse situation is equally striking in Patients 15 and 18 where there was marked improvement in icteric indices with only minimal improvement in erythrocyte fragility. Thus it would appear that though both values follow the same general trend the magnitudes of the changes are not related.

From these two observations it may be concluded that the erythrocyte fragility is not a function of the bile pigment concentration of the serum as measured by the icteric index. Nevertheless, in this disease there are other changes in serum chemistry (for example, cholesterol) which may be considered capable of altering the erythrocyte fragility. The laboratory facilities available to us at that time did not permit further chemical studies, but a direct approach to this problem proved enlightening. Normal washed erythrocytes were incubated in homologous serum from patients with acute infectious hepatitis, and conversely, washed cells of decreased fragility from a patient with hepatitis were incubated in normal homologous serum. In Table II it is demonstrated that a three-hour incubation at 38° C. failed to cause a significant change in fragility of either the normal cells or the cells from the patient with

hepatitis. It may be concluded that the decrease in erythrocyte fragility seen in acute infectious hepatitis is not caused by the direct action of any chemical component of icteric serum on normal erythrocytes. The implication that there is some intrinsic defect in these erythrocytes is substantiated by the fact that these low fragility (abnormal) cells from patients with acute infectious hepatitis failed to return to normal when incubated in normal serum. The possibility demonstrated by Haden[7] that this decreased fragility during jaundice is due to a decrease in the ratio of thickness to diameter of the erythrocytes was not investigated but would seem to supply an explanation for the fragility change.

TABLE II

	BEFORE INCUBATION		AFTER INCUBATION	
	INITIAL HEMOLYSIS	COMPLETE HEMOLYSIS	INITIAL HEMOLYSIS	COMPLETE HEMOLYSIS
Normal cells placed in serum with icteric index of 122	44	32	44	33
Cells of decreased fragility (from a patient with hepatitis) placed in normal serum	37	23	38	23

Further evidence supporting the hypothesis that the decreased erythrocyte fragility is due to an intrinsic malformation of the cell, or to failure in erythropoiesis, is seen in the increased fragility span in infectious hepatitis (Fig. 3, Curve C). While some cells maintain a normal high degree of fragility, so that the curve of initial hemolysis falls only slightly below normal, other cells are produced which have a strikingly reduced fragility, causing the levels of complete hemolysis to fall precipitously. These disproportionate changes in initial and complete hemolysis cause an increase in fragility span, a manifestation of an increased heterogenicity in the cell population. If decreased erythrocyte fragility were due to the direct action of an extrinsic factor (such as bile pigment or cholesterol) on cells that are otherwise normal, it could be assumed that all cells would undergo an equal decrease in fragility. Initial and complete hemolysis levels would fall the same extent, and the observed increased fragility span would not have been anticipated.

Thus, all the evidence points away from the direct action of some extrinsic factor in the serum and toward a faulty erythropoiesis as being responsible for the decreased erythrocyte fragility in acute infectious hepatitis. It is conceivable that diffuse liver damage may cause this fault in erythropoiesis. If this interpretation is correct, changes in liver function are manifested by altered erythrocyte fragility; the results of this simple test, which have been found to be consistently abnormal in acute infectious hepatitis, may serve as another index of the degree of liver damage.

CONCLUSIONS

1. The fragility of erythrocytes in acute infectious hepatitis is consistently decreased.

2. Evidence is presented which indicates that this decreased fragility is not a function of the degree of jaundice.

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THE TREATMENT OF MACROCYTIC ANEMIA WITH LACTOBACILLUS CASEI FACTOR (PTEROYLGLUTAMIC ACID)

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SINCE *Lactobacillus casei* factor (folic acid) was synthesized in 1945 a number of reports have appeared indicating the usefulness of this vitamin in the treatment of human macrocytic anemia. Darby, Jones, and Johnson,[1] Spies and co-workers,[2] Moore and associates,[3] Goldsmith,[4] and Doan, Wilson, and Wright[5] have found *L. casei* factor to be effective in the treatment of sprue, pernicious anemia, nutritional macrocytic anemia, and the macrocytic anemia of pregnancy. The structure of *L. casei* factor has recently been elucidated and the name pteroylglutamic acid suggested for this compound.[6]

This report deals with the use of pteroylglutamic acid in fifteen persons with anemia, five of whom have been studied for more than six months. Two patients with pernicious anemia, four with nutritional macrocytic anemia, and one with normocytic anemia in which the bone marrow was depressed showed marked clinical and hematologic improvement when *L. casei* factor was given as the sole therapeutic agent. Two patients with sprue and one with a celiac syndrome improved in certain respects, but the blood picture was altered in only one instance. *L. casei* factor was without beneficial effect in two patients with aplastic anemia and in one patient each with macrocytic anemia associated with regional ileitis and myxedema and macrocytic anemia of unknown origin.

METHOD OF STUDY

Patients with anemia were hospitalized for the first month or more of study and in most instances were placed on diets low in protein and containing no meat. Complete blood counts were obtained daily at first and later at increasing intervals. Blood was obtained by venipuncture without stasis, and approximately 5 c.c. were placed in tubes containing ammonium and potassium oxalate. United States Bureau of Standards equipment was used for all blood counts. Hemoglobin was determined by the Newcomer method, and the volume of packed erythrocytes was measured with the Wintrobe hematocrit tube. Reticulocyte stains were made with the wet film technique. Other laboratory determinations in this study included urinalyses, serologic tests for syphilis, gastric analyses after histamine stimulation, stool examinations, glucose tolerance tests, roentgenologic examinations of the chest and gastrointestinal tract, bone marrow biopsies, and renal function and vitamin excretion tests.

L. casei factor was administered either orally or parenterally in amounts which varied from 5 to 120 mg. daily. A few patients who failed to respond satisfactorily to *L. casei* factor were given pyridoxine, calcium pantothenate, iron, or liver extract as additional therapy.

The clinical condition of each patient was checked at frequent intervals, and laboratory procedures were repeated as often as seemed advisable. The period of time during which patients have been followed has ranged from four weeks to more than six months. One patient has been studied at intervals for over two years.

From the Nutrition Research Laboratory of the Department of Medicine, Tulane University School of Medicine, and the Charity Hospital of Louisiana at New Orleans.

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RESULTS

Some of the pertinent laboratory and clinical findings in the fifteen patients with anemia who were treated with *L. casei* factor are given in Table I. The diagnoses which were made as to type of anemia are also indicated. The criteria which were used in diagnosing pernicious anemia were those which are generally accepted, including absence of free hydrochloric acid from the gastric contents after histamine stimulation, a macrocytic anemia without demonstrable primary cause, and a hyperplastic bone marrow containing large numbers of megaloblasts. Good nutrition, elevation of serum bilirubin, and mild neurologic changes were usually present.

The diagnosis of sprue was made when there was steatorrhea, loss of weight, a macrocytic anemia with bone marrow findings similar to pernicious anemia, a flat glucose tolerance test, and a deficiency pattern on x-ray examination of the small intestine. Pigmentation of the skin and gastric achlorhydria were found in one patient.

The term nutritional macrocytic anemia was applied to patients who had been receiving inadequate diets and in whom macrocytic anemia occurred in association with symptoms and signs of deficiency of vitamins of the B complex. Diarrhea, glossitis, and loss of weight were constant findings. Free hydrochloric acid was often present in the gastric juice after histamine stimulation. Fat in the stools was not increased. The bone marrow varied, being either hypercellular or depressed, and normoblasts or pronormoblasts were the predominant cell in some instances, rather than megaloblasts. Macrocytosis was usually less prominent than in pernicious anemia of similar severity. Anemia of nutritional origin may respond to yeast or to a good diet without special medication.

The seven patients with macrocytic anemia whose condition improved with the administration of pteroyl glutamic acid responded to treatment in a characteristic manner. Reticulocytes in the blood increased at the end of two to six days reaching a peak after five to eleven days. The rise was not as great as would have been anticipated had liver extract been administered, varying from 5 to 12 per cent. Similar findings were recently reported by Heinle, Welch, and Nelson.[7] The increase in reticulocytes often persisted for long periods of time especially if large amounts of *L. casei* factor were given daily. The total erythrocyte count and the percentage of hemoglobin began to rise within a week to ten days, the maximum being attained in twenty-four to forty-three days when the amount of *L. casei* factor given was 15 mg. daily. The initial leucocyte count was low in three instances and rose to normal in seven to fourteen days after therapy had been started. The number of platelets likewise increased. Four patients have been maintained in good clinical condition and with a relatively normal blood picture for over six months.

In four instances (Patients 3 to 5 and 7) the bone marrow was examined both before and after therapy with *L. casei* factor. Regardless of whether the initial findings were those of depression or stimulation there was a return to normal after six to eight weeks of treatment.

Findings in Pernicious Anemia.—The diagnosis of pernicious anemia was made in two patients. The first was a white woman, 55 years of age, who gave

TABLE I. CLINICAL AND LABORATORY FINDINGS IN PATIENTS WITH ANEMIA PRIOR TO THERAPY

PATIENT	RACE	SEX	AGE	TYPE OF ANEMIA	BLOOD PICTURE					BONE MARROW	FREE HCl	DIET	OTHER FINDINGS
					R.B.C. (MILLIONS)	HB. (GM.)	HCT.* (%)	MCV†	MCHC‡				
1	W	F	55	Pernicious	1.7	6.2	20.5	121	30	Hypercellular megaloblasts	0	Good	Numbness, fingers
2	W	M	57	Pernicious	3.7	11.0	37.0	100	30		0	Fair	Numbness; vibratory sense and reflexes ↓ legs
3	C	M	40	Undetermined (pernicious?)	1.7	4.9	14.0	82	35	Depressed, few megaloblasts	0	Fair	Numbness, fingers
4	W	F	68	Nutritional macrocytic	2.3§	7.0	22.0	96	32	Hypercellular pronormoblasts	0	Very poor	Emaciation, diarrhea, stupor
5	W	F	67	Nutritional macrocytic	1.8	6.0	20.0	111	30	Cellular, erythroid series depressed, normoblasts, pronormoblasts	55	Poor	Weight loss, edema, diarrhea, mental confusion
6	W	F	64	Nutritional macrocytic	2.3	8.4	29.0	126	35	Cellular, no megaloblasts	0 18	Very poor	Edema, polyneuritis, emaciation, diarrhea, irritability
7	W	F	69	Nutritional macrocytic	1.7	5.0	15.0	88‖	33	Depressed, many megaloblasts	26	Poor	Numbness
8	W	F	55	Sprue	3.5	11.5	39.0	111	29			Fair	Steatorrhea, weight loss
9	W	F	54	Sprue	3.6	11.3	38.0	106	30	Hypercellular pronormoblasts, normoblasts	0	Fair	Diarrhea, stool fat increased
10	W	M	4	Celiac syndrome	3.9	11.0	38.0	97	29			Poor	Emaciation, steatorrhea
11	C	M	63	Undetermined	2.1	6.8	26.0	124	27	Hypercellular pronormoblasts, normoblasts	0	Very poor	Emaciation Combined sclerosis
12	C	M	51	Aplastic	0.8	2.8	10.0	125	28	Depressed lymphocytes predominant	0	Fair	Glossitis minimal
13	W	M	8	Aplastic	2.2§	5.6	18.0	82	31	Depressed, few normoblasts	20	Fair	
14	W	F	50	Myxedema	3.2	10.2	32.0	100	32			Good	
15	W	M	30	Regional ileitis	3.4	10.2	35.0	103	29			Good	Diarrhea

All patients had glossitis except Patients 11, 14, and 15.

*Hct., Hematocrit-volume packed erythrocytes.

†MCV, Mean corpuscular volume.

‡MCHC, Mean corpuscular hemoglobin concentration.

§After transfusions.

‖MCV has been above normal during most of period of study.

a history of dyspnea, palpitation, and dizziness for six months prior to entering the hospital in November, 1945. She had diarrhea and vomiting at intervals and had lost weight, although she was still obese. Weakness, numbness of the fingers, and a yellowish discoloration of the skin had been present for one month. The patient stated that her sister was being treated for anemia with liver extract. Physical examination showed moderate obesity, a pale lemon-yellow skin, a smooth red tongue with atrophic papillae, slight cardiac enlargement, and a systolic murmur audible in all cardiac valve areas. Neurologic examination was

TABLE II. HEMATOLOGIC RESPONSE OF PATIENTS WITH PERNICIOUS ANEMIA TO *L. Casei* FACTOR

PATIENT	DAYS AFTER BEGINNING THERAPY	R.B.C. (MILLIONS)	HB. (GM.)	HCT.* (% R.B.C.)	AMOUNT *L. Casei* FACTOR PER DAY	DIET
1	0	1.7	6.2	20.5	15 mg. I.M.,† 20 days	Low protein, meat free
	31‡	3.5	10.0	35.0	None	Low protein, meat free
	47	3.1	9.5	36.0	15 mg. Orally	Low protein, meat free
	90‡	4.0	12.0	40.0	15 mg. Orally	Regular
	160	4.0	11.2	40.0	15 mg. Orally§	Regular
	188	4.3	11.9	41.0	15 mg. Orally	Regular
2	0	3.5	10.5	36.0	120 mg. Orally, 6 days	Low protein
	9	4.0	11.9	39.0	30 mg. Orally	Low protein
	24	4.2	13.5	44.0	30 mg. Orally‖	Regular
	56‡	5.8	18.9	48.0	30 mg. Orally	Regular
	84	6.0	16.6	5.5	5 mg. Orally	Regular
3	0	1.7	4.9	14.0	15 mg. I.M.,† 19 days	Regular
	43‡	4.0	12.0	39.5	None	
	57	3.5	9.4	39.0	None‖	
	106	4.5	11.5	41.0	None	
	169	3.0	9.6	31.0	None	
	190	2.5	7.8	29.0	None	

*Hct., Hematocrit.
†I.M., Intramuscularly.
‡Maximum rise with given dose of *L. casei* factor.
§Ferrous sulfate added, 1 Gm. daily.
‖Left hospital.

normal. The icterus index was 20, while other laboratory findings, except for the anemia, were within normal limits. This patient was placed on a low protein, meat-free diet and was given 15 mg. of *L. casei* factor daily, intramuscularly, for twenty days. The hematologic response is shown in Fig. 1. After forty-seven days it was apparent that no further improvement would occur, and she was given *L. casei* factor orally, 5 mg. three times daily. This medication has been continued for 188 days (Table II). At the end of three months a regular diet was instituted. This had no effect on the blood count which remained slightly below normal with macrocytosis persisting. Iron was added to the therapeutic regimen, and after four weeks improvement of questionable significance occurred. During the seven months in which this patient has been observed she has gained weight and strength, the lingual papillae have regenerated, and numbness of the hands has disappeared. She is without complaints and is working daily in a newspaper office.

MCV, Mean corpuscular volume.
‡MCHC, Mean corpuscular hemoglobin concentration.
§After transfusions.
‖MCV has been above normal during most of period of study.

The second patient with pernicious anemia was a 57-year-old white man who was treated with liver extract from April, 1944, to December, 1945, at which time he discontinued therapy. He returned to the hospital in April, 1946, complaining of weakness, numbness of the hands and feet, and soreness of the tongue. In addition to a mild macrocytic anemia the positive findings on examination were a pale, smooth tongue, absence of vibratory sensation and

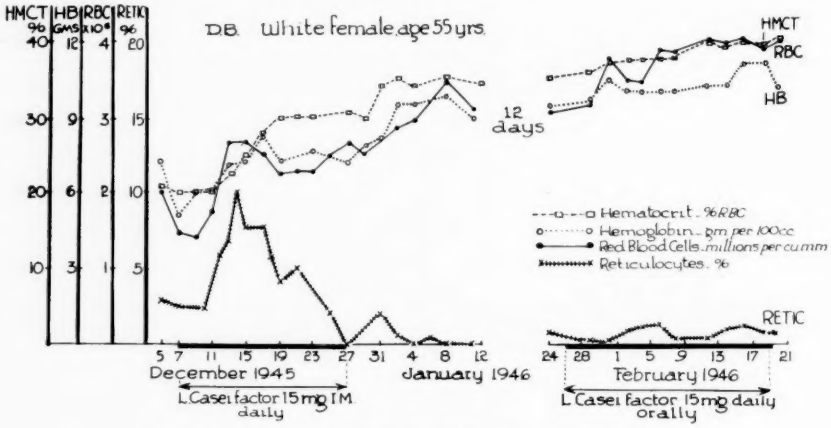

Fig. 1.—Pernicious anemia treated with *L. casei* factor.

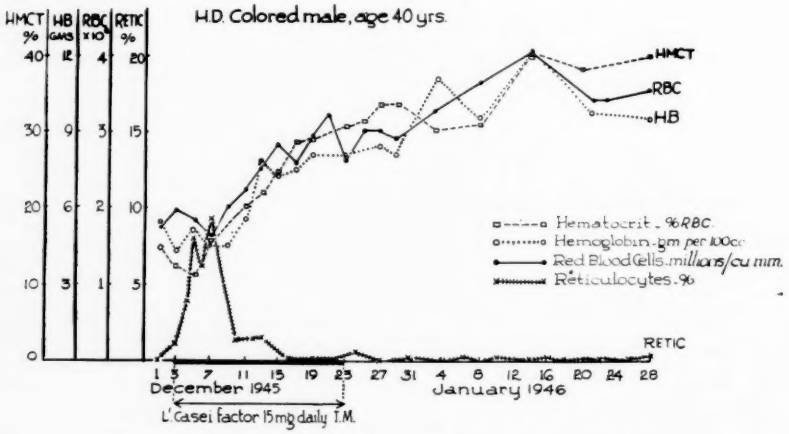

Fig. 2.—Hypoplastic anemia treated with *L. casei* factor.

diminished reflexes in both lower extremities, achlorhydria following histamine administration and atrophic gastritis involving the pyloric antrum, and pars media of the stomach on gastroscopic examination. The initial blood picture and the response to therapy with *L. casei* factor is shown in Table II. In this patient a very marked increase in both erythrocytes and hemoglobin has occurred. This is the only instance in which high normal levels have been attained. Whether or not the massive dose of *L. casei* factor which was used is responsible for this finding will require further study. The patient feels well, numbness and

weakness have disappeared, reflexes in both legs are normal, and vibratory sensation has returned in the left lower extremity.

The third patient included in this group was at first thought to have aplastic anemia. However, the subsequent course suggests that he, too, has pernicious anemia. He is a colored man, 40 years of age, who entered the hospital in November, 1945, complaining for the past eight weeks of weakness, dyspnea, and substernal distress on exertion. In 1943 he was treated for amebiasis. At that time a severe anemia was discovered but therapy was not instituted. Physical examination in 1945 showed a well-nourished Negro with pallor of the skin and mucous membranes. The tongue was atrophic. There was a faint systolic murmur audible in all cardiac valve areas and the liver was palpable. Several gastric analyses showed achlorhydria. Other laboratory findings including x-ray examination of the chest and gastrointestinal tract were normal, except for anemia which was normocytic and normochromic in type. The bone marrow was markedly depressed; lymphocytes predominanted but a few megaloblasts were seen. It was suggested that these findings might represent pernicious anemia in an aplastic phase. *L. casei* factor was administered intramuscularly in amounts of 15 mg. daily for nineteen days. The response to therapy is illustrated in Fig. 2. No further treatment has been given, and the blood count has decreased. Although he feels well at the present time, the patient has a macrocytic anemia of moderate severity (Table II).

Findings in Nutritional Macrocytic Anemia.—Four patients with nutritional macrocytic anemia were treated with *L. casei* factor (Patients 4 to 7). All were white women over 60 years of age who had been eating very limited diets, inadequate in calories and in vitamins of the B complex. Each patient complained of anorexia, soreness of the tongue, diarrhea, and loss of weight. Three patients had free hydrochloric acid in the stomach. Bone marrow findings were variable (Table I). Vitamin excretion tests in each of the four patients showed a subnormal output of thiamine, niacin, and riboflavin.

Patient 4 was extremely weak and emaciated when admitted to the hospital in October, 1945. She had been vomiting for two weeks, was dehydrated and semistuporous, and appeared much older than her actual age of 68. She was incontinent of both urine and feces. The skin was dry and pale. The tongue was red, smooth, and atrophic, and only three carious teeth remained in the mouth. The heart was slightly enlarged to the left, and the electrocardiogram showed low QRS complexes, a prolonged QT interval, and inverted T waves in Lead I. The erythrocyte count on admission was 700,000 per cubic millimeter with an hematocrit reading of 8.5 per cent. Serum proteins were reduced to 5.5 Gm. per 100 c.c. of blood. Other laboratory findings were not remarkable. The patient received three transfusions, a high caloric diet, thiamine, niacin, and riboflavin. She became mentally clear, gained strength, and sphincter control returned. The tongue was unimproved. After a month, this therapy was discontinued and she was placed on a low protein diet. *L. casei* factor was administered in amounts of 15 mg. daily intramuscularly for twenty days. The anemia improved as shown in Table III. The papillae of the tongue regenerated

rapidly. Several months later *L. casei* factor was given orally, 10 mg. three times daily. The blood count at the end of two months of treatment with this large amount of *L. casei* factor is essentially normal. The patient feels well, has gained 35 pounds, and all symptoms of anemia have disappeared.

Patient 5 had noted weakness and swelling of the ankles for nine months before entering the hospital. At the time of admission she was confused, semi-stuporous, and the rectal temperature was 102° Fahrenheit. Blood pressure was 180/60, pulse rate 110. The mouth was edentulous and the tongue purplish red and atrophic. There was slight cardiac enlargement, a blowing systolic murmur in all of the valve areas, occasional premature contractions, râles at the base of the left lung, ascites, and edema of the legs. The calf muscles were extremely tender. Venous pressure and circulation time were slightly elevated. A glucose tolerance test showed a flat curve; serum proteins were normal.

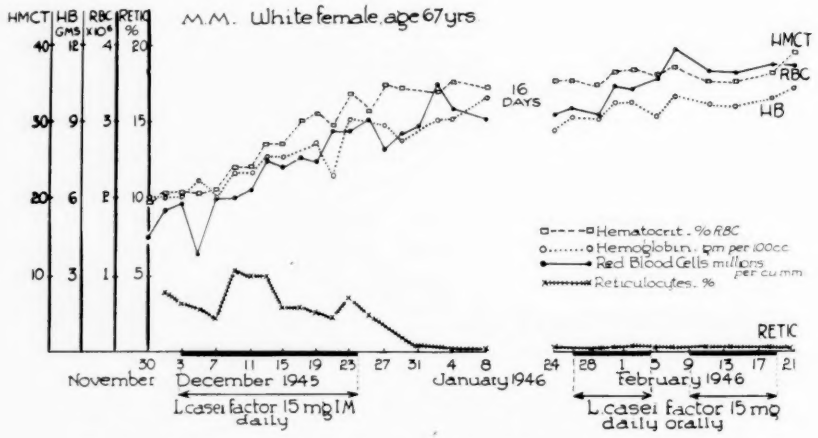

Fig. 3.—Nutritional macrocytic anemia treated with *L. casei* factor.

Liver function as measured by the hippuric acid and cephalin flocculation tests was normal. The urine contained 3 per cent moist albumen. The patient was digitalized and treatment with *L. casei* factor was instituted. The hematologic response to 15 mg. given parenterally daily is shown in Fig. 3. From a clinical standpoint there was rapid improvement. The patient developed a ravenous appetite, gained in weight and strength, and papillae of the tongue regenerated. The edema gradually disappeared and cardiac compensation was restored. Subsequent therapy and the changes which occurred in the blood picture are shown in Table III. *L. casei* factor was effective with either parenteral or oral administration, but the blood count remained below normal. Increasing the amount of *L. casei* factor to 30 mg. a day has not resulted in further improvement.

Patient 6 was first seen in May, 1945, at which time she complained of weakness and burning of the tongue of five months' duration and a loss in weight of 55 pounds. For three months she had noted paresthesias of the toes and for one month diarrhea, three or four watery stools daily, and edema of the

ankles. Examination showed undernutrition, glossitis, a macrocytic anemia of mild degree, and tenderness of the calf muscles. A diagnosis of deficiency of the vitamin B complex was made, and treatment with thiamine, niacin, riboflavin, and brewers' yeast was instituted. After one month the patient was discharged from the hospital much improved.

The patient returned in April, 1946, at which time she was so weak she could hardly stand. She had noted a sore tongue, diarrhea, dyspnea on excretion, edema of the ankles, and painful feet. She was irritable and did not cooperate well. Her diet for five months had consisted largely of rice, grits, and gravy. She weighed 78 pounds. The tongue was red and no papillae were visible. Cheilosis with angular maceration was present. There was definite evidence of peripheral neuritis involving both lower extremities which were also edematous. The heart and lungs were normal. Blood pressure was 95/60.

TABLE III. HEMATOLOGIC FINDINGS IN PATIENTS WITH NUTRITIONAL MACROCYTIC ANEMIA TREATED WITH *L. Casei* FACTOR

PATIENT	DAYS AFTER BEGINNING THERAPY	R.B.C. (MILLIONS)	HB. (GM.)	HCT.* (% R.B.C.)	AMOUNT *L. Casei* FACTOR PER DAY	DIET
4	0	2.3	7.0	22.0	15 mg. I.M.,† 20 days	Low protein, meat free
	24‡	4.5	11.0	39.0	None	High vitamin, high caloric
	103	3.3	10.0	36.0	30 mg. Orally	
	126	3.7	11.6	42.0	30 mg. Orally§	
	179	4.4	14.9	46.0	5 mg. Orally	
	200	4.6	13.0	46.0	5 mg. Orally	
5	0	1.8	6.0	20.0	15 mg. I.M.,† 20 days	Low protein, meat free
	30‡	3.4	9.0	34.0	None	Regular
	54	3.2	9.0	34.5	15 mg. Orally, 19 days	Regular
	93	4.0	10.7	38.0	15 mg. Orally§	
	154	3.9	10.8	38.0	30 mg. Orally	
	189	4.2	10.4	37.0	30 mg. Orally	
6	0	2.3	8.4	29.0	40 mg. I.M.,† 3 times weekly	High protein
	35‡	3.6	10.7	38.0	40 mg. I.M.,† 3 times weekly	
	60	3.7	9.9	37.0	100 mg. Orally, 7 days	High protein
	84	4.0	9.9	37.0	None	

*Hct., Hematocrit.
†I.M., Intramuscularly.
‡Maximum rise with given dose of *L. casei* factor.
§Left hospital.

Roentgenologic examination of the gastrointestinal tract was without abnormal findings. There was achlorhydria after histamine administration and serum proteins were reduced (5.46 Gm. per cent). Liver function tests were normal. There was a moderately severe macrocytic anemia. *L. casei* factor was administered in amounts of 40 mg. intramuscularly three times weekly, and a high protein diet was instituted. The anemia improved (Table III) but a completely normal blood picture was not attained. After receiving 100 mg. of *L. casei* factor orally every day for one week there was only a slight additional rise in the blood count. In spite of the persistence of mild anemia the patient

has improved remarkably. Edema has disappeared, papillae of the tongue have regenerated, and cheilosis has healed. Thiamine was administered in amounts of 10 mg. three times daily after the first two weeks in the hospital, and there has been complete recovery from the peripheral neuritis. The patient has gained 28 pounds.

Patient 7 has been followed for more than two years. When first seen in October, 1943, she complained of anorexia, diarrhea, and vomiting for six months. She had lost 70 pounds. She was undernourished, pale, edentulous, and had an atrophic tongue. The heart was not enlarged. There was a soft blowing apical systolic murmur and occasional premature contractions. Peripheral blood vessels were extremely sclerotic. She had a macrocytic anemia, leucopenia, and thrombopenia. There was free acid in the stomach. Extensive investigation showed no evidence of malignancy, infection, or disease of the liver. Since 1944 this patient has been treated at intervals with various antianemic substances. In April, 1944, 5 mg. of folic acid concentrate and 100 of calcium pantothenate were given daily for one week. Reticulocytes increased to 12 per cent on the ninth day; this was followed by an increase in erythrocytes and hemoglobin (Table IV). Folic acid concentrate was given

TABLE IV. HEMATOLOGIC FINDINGS IN PATIENT WITH NUTRITIONAL MACROCYTIC ANEMIA TREATED WITH "FOLIC ACID" AT INTERVALS FOR TWENTY-SIX MONTHS

	BLOOD PICTURE			
DATE	R.B.C. (MILLIONS)	HB. (GM.)	HCT.* (% PACKED R.B.C.)	MEDICATION† (AMOUNT GIVEN DAILY)
4/23/44	1.7	5.0	15.0	Folic acid concentrate,‡ 5 mg., and calcium pantothenate, 100 mg.; 4/23/44 to
5/ 8/44	2.5	7.5	26.0	
5/29/44	2.7	7.5	28.0	4/30/44
10/ 7/44	3.3	11.5	34.5	Folic acid concentrate,‡ 5 mg.; 10/7/44
10/28/44	4.1	13.0	39.0	to 10/14/44
6/ 8/45	2.1	9.1	28.5	Crystalline folic acid, 5 mg., and calcium
7/12/45	3.0	11.5	36.0	pantothenate, 10 mg., 6/8/45 to 6/15/45
10/19/45	2.3	9.5	24.5	*L. casei* factor, 5 mg.; 10/19/45 to 10/28/45
11/ 9/45	2.5	10.5	31.0	*L. casei* factor, 5 mg., and calcium pantothenate, 10 mg.; 11/9/45 to 11/18/45
11/23/45	2.9	11.5	34.5	*L. casei* factor, 15 mg., intramuscularly; 11/23/45 to 11/29/45
12/ 1/45	4.2	10.5	35.0	
12/17/45	3.0	11.5	37.5	
1/20/46	3.1	10.0	38.0	*L. casei* factor, 15 mg.; 1/20/46 to 2/10/46
2/ 9/46	4.1	10.2	37.5	
2/20/46	3.4	10.5	37.5	
3/18/46	3.9	11.0	37.5	*L. casei* factor, 30 mg.
4/22/46	4.0	11.0	38.5	*L. casei* factor, 30 mg.
5/27/46	4.1	12.0	39.0	*L. casei* factor, 30 mg., and pyridoxine, 30 mg.
6/24/46	4.2	11.3	39.0	*L. casei* factor, 30 mg., and pyridoxine, 30 mg.

*Hct., Hematocrit.
†Given orally except 11/23/45 to 11/29/45.
‡"Fermentation" folic acid.

again in October, 1944. A slight improvement in the blood picture occurred. Crystalline folic acid was followed by a similar hematologic response in June, 1945. Since synthetic *L. casei* factor has been available it has been administered in varying dosage on several occasions, either alone or in combination with calcium pantothenate or pyridoxine. In most instances there has been hematologic improvement with *L. casei* factor, although large doses have not brought about an increase in erythrocytes above 4 to 4.2 million or of hemoglobin above 11 to 12 grams. It is of course possible that this is a normal blood picture for this patient. In these studies there is no definite evidence that calcium pantothenate or pyridoxine influenced blood regeneration.

This patient has been treated also with liver extract, yeast, iron, and individual vitamins of the B complex. The hematologic changes over the period of one and one-half years with various medications are shown in Fig. 4. Oral liver extract given after the administration of folic acid concentrate was without effect on the blood picture, while concentrated liver extract given parenterally brought about a marked rise in erythocytes and hemoglobin. On one occasion brewers' yeast, three ounces daily, was given for five weeks without effect. There was no improvement with 1 Gm. of ferrous sulfate daily for two months or with 15 mg. thiamine, 150 mg. niacin, and 5 mg. riboflavin. daily for one month.

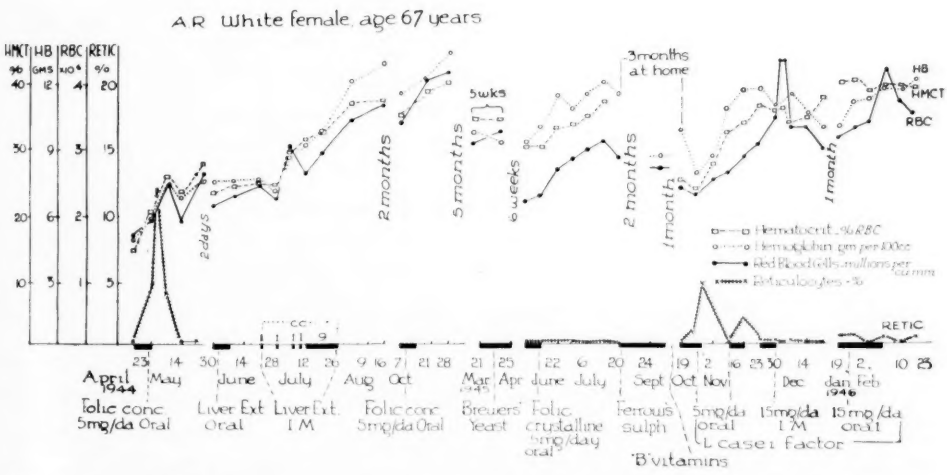

Fig. 4.—Hematologic response to various therapeutic agents in a patient with nutritional macrocytic anemia. (April 24, 1944, through Feb. 23, 1946.)

Findings in Sprue.—Two patients with sprue have been treated with *L. casei* factor. Patient 8 (Table I) has been known to have sprue for fourteen years. In 1932 at the time the original diagnosis was made, her weight had decreased from 150 to 55 pounds. She had steatorrhea, abdominal distention, glossitis, ulcers of the mouth, profound anemia of macrocytic type, and weakness so marked that she could scarcely move in bed. Since that time she has been eating a diet high in protein and bananas and has received liver extract. Whenever liver has been omitted the blood count has fallen and symptoms of anemia have

reappeared. The steatorrhea has never been completely controlled. Even when the blood count was high (erythrocytes 4.5 to 5 million) she has had occasional bulky, frothy stools containing large amounts of fat and fatty acid. In April, 1945, liver extract was discontinued, the diet remaining constant. By January, 1946, she had lost 7 pounds, stools had become increasingly fatty, the tongue had become sore, and the blood count had fallen slightly (Table I). Roentgenologic examination of the small intestine showed a typical deficiency pattern. She felt weak and was unwilling to remain without therapy for a longer period of time. *L. casei* factor, 50 mg., was given daily by mouth, and meat was withdrawn from the diet, which did, however, include bananas and milk. Within three days she felt stronger. The bowels improved markedly and she had only one large stool daily which contained less fat. Soreness of the tongue was relieved and papillae reappeared on the sides and tip. The patient regained her lost weight. The blood count showed no change at the end of three weeks. *L. casei* factor was given intramuscularly, at first 20 mg., then 40 mg. three times a week for a total of five weeks. The blood count remained essentially the same. However, the percentage of reticulocytes which had been 0.2 to 2 before therapy ranged from 1 to 4 per cent when *L. casei* factor was administered. Meat was added to the diet without any effect on the anemia. The addition of pyridoxine to the regimen was also without benefit. At the end of three and one-half months, therapy with *L. casei* factor was discontinued and injections of concentrated liver extract (30 units) were given three times a week for four weeks. The percentage of hemoglobin and volume of packed red cells remained the same, but the erythrocyte count increased from 3.6 to 4 million per cubic millimeter. Reticulocytes varied from 4 to 6 per cent. This slight change suggests a decrease in macrocytosis.

The second patient with sprue (Patient 9) was first seen in August, 1945. She had a history of diarrhea of fatty type for two years, a loss of 15 pounds in weight in the previous four months, and burning of the tongue. A glucose tolerance test showed a flat curve, and there was histamine refractory achlorhydria. The stools contained large amounts of fat, and examination of the blood showed a macrocytic anemia. Marked improvement occurred with a diet high in protein, low in fat and carbohydrate, and injections of crude liver extract. Treatment was stopped in October, 1945, and in April, 1946, the patient had again developed fatty diarrhea, a sore tongue, and had lost weight. The blood count at that time is given in Table I. *L. casei* factor, 30 mg., was given orally daily for three weeks, at which time erythocytes were 3.8 million per cubic millimeter, hemoglobin 11.6 Gm., and the volume of packed erythrocytes 40.5 per cent. Calcium pantothenate, 30 mg. daily, was prescribed in addition to *L. casei* factor for another three weeks, at which time erythocytes were 3.9 million per cubic millimeter, hemoglobin 11.3 Gm., and the volume of packed red cells 40 per cent. Although the anemia was not significantly influenced by therapy, the diarrhea was controlled, soreness of the tongue disappeared, and the patient gained 6 pounds. Therapy was then changed to liver extract, 15 units being given three times the first week and 30 units three times a week for the subsequent two

weeks. Reticulocytes increased from 3 to 6 per cent, while erythrocytes and hemoglobin rose to 45 million per cubic millimeter and 12 Gm. per cent, respectively. The volume of packed red cells increased to 41 per cent.

One patient with a celiac syndrome was treated with *L. casei* factor (Patient 10). He was a 4-year-old white boy who was underdeveloped and emaciated and who had had steatorrhea since the age of two or three weeks. A diagnosis of pancreatic fibrosis was considered but not proved. Anemia was normocytic and normochromic and failed to improve with iron. When *L. casei* factor was administered the stools became less frequent and more formed. There was a slight gain in weight. Hematologic studies showed the following after thirteen daily injections of 20 mg. of *L. casei* factor; erythrocytes had increased from 3.9 to 4.5 million per cubic millimeter, hemoglobin from 10.9 to 12.5 Gm. per cent, and volume of packed red cells from 38 to 40.5 per cent. This change, while small, seemed definite.

Other Types of Anemia.—Two patients with aplastic anemia (Patients 12 and 13, Table I) received *L. casei* factor without benefit. Patient 13 was an 8-year-old white boy; Patient 12 was a colored man, 51 years of age. In neither instance could any cause of the anemia be discovered in spite of extensive investigation. In one instance (Patient 12) the anemia was macrocytic. This patient was given 5 mg. and then 30 mg. of *L. casei* factor daily. At the end of one month there had been no improvement and it had been necessary to give several blood transfusions. Patient 13 received 20 mg. of *L. casei* factor, parenterally daily for ten days, then 40 mg. daily for a similar period. There was no change in the blood picture, and seven blood transfusions were needed in a period of fifty days.

Patient 11 (Table I) had a macrocytic anemia, the cause of which could not be determined. On admission to the hospital, the tentative diagnosis was pernicious anemia with degeneration of the posterior and lateral columns of the spinal cord. The patient was an emaciated colored man who was extremely weak. There was loss of vibratory and position sensibility and absence of the deep reflexes in both lower extremities. The Babinski reaction was positive on the left, and there was loss of sphincter control. There was a macrocytic anemia which was hypochromic suggesting that iron deficiency was complicating the picture. The patient had no glossitis, and the bone marrow while hypercellular did not contain megaloblasts. Physical examination and numerous laboratory studies including spinal puncture and x-rays of the chest and gastrointestinal tract failed to show any evidence of neoplasm or infectious disease. *L. casei* factor was given in amounts of 15 mg. daily for two weeks without any effect. The patient developed a urinary tract infection and bed sores and died after one month in the hospital. An autopsy could not be obtained.

Patient 14 who had myxedema and a mild macrocytic anemia was given 30 mg. of *L. casei* factor daily for fifteen days without benefit, which finding was not unexpected. She also failed to improve with liver extract.

Patient 15 had regional ileitis with a mild anemia which had improved somewhat with therapy with liver extract. It was felt that *L. casei* factor might

benefit the mild macrocytic anemia which persisted; therefore, liver injections were discontinued and *L. casei* factor was given in 20 mg. doses intramuscularly at intervals for five weeks, a total of 500 mg. being administered. There was no change in the blood picture.

DISCUSSION

The preceding case reports indicate that *L. casei* factor (pteroylglutamic acid) is a potent hemapoietic substance in pernicious anemia and in anemia of nutritional origin in which macrocytosis is a feature. Clinical improvement paralleled hematologic response in all instances. A gain in weight and strength and relief of glossitis with regeneration of lingual papillae occurred. These findings are corroborative of those reported by other investigators. In addition, one patient with pernicious anemia had neurologic changes which have improved with therapy, although, as yet, complete return to normal has not been obtained.

Findings in this study differ slightly from those previously reported in that the reticulocyte response to *L. casei* factor was much less than would have been anticipated with liver extract. Also the mild anemia of two patients with sprue was unaffected by pteroylglutamic acid, although improvement in other respects was noted. Liver extract, administered subsequently, was without effect on the blood picture of one of these patients but led to improvement in the other. Iron, in one instance, was not beneficial.

In previous studies *L. casei* factor has been administered only to patients in whom the bone marrow was hyperplastic and contained megaloblasts. In two patients in this series the bone marrow was depressed, and yet marked hematologic improvement occurred. In both instances, megaloblasts were the predominant cell of the hypoplastic marrow. Hematologic response also occurred with a cellular marrow in which pronormoblasts or normoblasts predominated. *L. casei* factor was not beneficial in aplastic anemia. Differentiation of anemias in which the bone marrow is depressed into those which will or will not respond to *L. casei* factor is difficult. If nutritional deficiency is present, or if megaloblasts are seen in the bone marrow, therapy with *L. casei* factor should be given a trial.

Eight patients have been treated continuously with *L. casei* factor for several months. In two the blood picture has become essentially normal (Patients 2 and 4). In the other six (Patients 1, 5 to 9) the hemogram has remained below the average normal,[8] with erythrocytes varying from 3.9 to 4.3 million per cubic millimeter and the hemoglobin from 9.1 to 11.0 Gm. per 100 cubic centimeters. The volume of packed red blood cells has ranged from 36 to 41 per cent which is essentially normal. A deficiency of iron may account for some of these findings, and therapy with ferrous sulfate has been started in several patients. The amount of *L. casei* factor administered does not account for the subnormal blood picture, as seven of the eight patients received 30 mg. or more daily at some time during treatment. This large quantity does not appear to be more effective than 15 mg. daily. In all likelihood the smallest quantity required to produce a maximal response will be found to be less than

15 mg. daily in most patients, with an even smaller amount needed for maintenance. Several patients are being treated at the present time with 5 mg. daily.*

Very little difference has been noted between the response of patients to *L. casei* factor and to liver extract other than the finding of a smaller rise in reticulocytes with the former, in most instances, and the observation that *L. casei* factor is equally effective with oral or parenteral administration. The anemia of one patient with sprue in this series of cases responded to liver extract but not to *L. casei* factor. As Moore and associates[3] have indicated, the hemapoietic effect of liver extract cannot be attributed solely to the content of *L. casei* factor. Some extracts of liver contain very little, far less than enough to produce therapeutic effect. Also, crystalline material obtained from liver extract has produced maximal reticulocyte response in pernicious anemia in relapse when given in quantities of only 0.035 mg. daily.[9] Thus, while pteroylglutamic acid and the erythrocyte maturing factor in liver extract produce similar effects, they appear to differ chemically and the mechanism of action of each requires elucidation.

SUMMARY

L. casei factor (pteroylglutamic acid) has been administered to fifteen patients with anemia in amounts of 5 to 120 mg. daily. Three patients with pernicious anemia and four with nutritional macrocytic anemia improved clinically and hematologically. There was a rise in reticulocytes and an increase in erythrocytes and hemoglobin which reached normal levels in two patients and remained slightly below normal in five. Leucocytes and platelets rose to normal in each instance in which initial values were low. In two patients who responded satisfactorily to pteroylglutamic acid, the bone marrow was depressed rather than hyperplastic and returned to normal within four to six weeks after therapy was instituted.

Two patients with sprue improved clinically, but the mild anemia which was present in each was unaffected by pteroylglutamic acid. The administration of liver extract failed to influence the anemia in one instance but was followed by improvement in the other. One child with a celiac syndrome was benefited slightly from both a clinical and hematologic standpoint when *L. casei* factor was administered.

L. casei factor was ineffectual in the treatment of two patients with aplastic anemia and of one patient each with macrocytic anemia associated with myxedema and regional ileitis and macrocytic anemia of unknown origin.

One patient with pernicious anemia and three patients with nutritional macrocytic anemia have been maintained in excellent condition for more than six months with pteroylglutamic acid as the therapeutic agent.

The author wishes to express appreciation to Dr. Pizzaloto, for his cooperation in obtaining bone marrow biopsies, to Miss Alma Lonsdale and Miss Betty Quinn, for technical assistance, and to Dr. G. Van Langerman and members of the intern and resident staffs of the Tulane Service at Charity Hospital, for their generous assistance and cooperation in this investigation.

*One patient with pernicious anemia in whom the initial erythrocyte count was 980,000 per cubic millimeter has shown a reticulocyte response of 42 per cent with 5 mg. pteroylglutamic acid daily.

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STUDIES IN IRON TRANSPORTATION AND METABOLISM

V. Utilization of Intravenously Injected Radioactive Iron for Hemoglobin Synthesis, and an Evaluation of the Radioactive Iron Method for Studying Iron Absorption

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THE data to be reported herein were obtained in a systematic study of the utilization of iron for hemoglobin synthesis. Observations were made both in human subjects and in dogs, under normal conditions and in the presence of various types of anemia. The method used was essentially that developed by Hahn and associates.[1] Tracer doses of radioactive iron (Fe^{59})* were injected intravenously, and the amount which later appeared in the peripheral blood as newly formed hemoglobin was measured.[2] In this way, both the rate and completeness of iron utilization were followed. It soon became apparent, however, that exact interpretation of results was frequently difficult because of variations in the rate of red blood cell formation and destruction. For instance, subjects with simple iron deficiency built all of the parenterally administered isotope into hemoglobin within six to nine days, while patients whose iron deficiency was associated with fever or a chronic debilitating disease used the iron more slowly and less completely. In these subjects, it was impossible to differentiate between an abnormality in iron utilization and some other disturbance in erythrocyte or hemoglobin formation.[3, 4] Patients with hypoplastic anemia used very little of the injected radioiron for hemoglobin synthesis, presumably because they were forming few new red blood cells. This same relationship was even more clearly demonstrated in observations on patients with untreated pernicious anemia. Radioactive iron appeared slowly in the circulating hemoglobin of these subjects until liver extract was injected; its utilization was then sharply accelerated and eventually became complete. Increased rates of red blood cell destruction also influenced results. In patients with hemolytic anemia, the radioiron appeared very promptly in the circulating hemoglobin, but the concentration of radioactivity quickly leveled off at values usually much less than 100 per cent of the injected amount. Iron in these subjects was certainly being utilized at a rapid rate, and failure to recover all of the radioiron in circulating hemoglobin could not be interpreted as indicating poor utilization. As will be emphasized later, the results in hemolytic anemias are compatible with the thesis that recently stored iron is more easily mobilized than iron stored for longer periods and is drawn upon for daily needs.[5, 6] When iron utilization is studied by the radioactive iron technique, therefore, several

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*Radioactive iron, the radioactive isotope of iron, and tagged iron are used as synonyms.

variables are involved. While interpretation of results is consequently more difficult and complicated, the results are of considerable interest.

In addition to providing fundamental information about both iron utilization and rates of hemoglobin synthesis, the results suggest that a re-evaluation should be made of the radioactive iron technique as a method of measuring iron absorption from the gastrointestinal tract. With this method, the amount of radioactive iron which appears in the total circulating blood within five to seven days after oral administration of the isotope is determined, and the ratio of this value to the total quantity of tagged iron ingested is taken as a measure of the amount absorbed.[1, 7-13] Sufficient emphasis has not been given to the fact that the method measures only that portion of the absorbed iron which is utilized for hemoglobin synthesis. In experiments with normal and anemic dogs, sacrificed at varying times after radioiron had been fed, Hahn and co-workers[1] were able to demonstrate very little radioactive iron retained in the tissues. The assumption that iron utilized was equivalent to the amount absorbed was, therefore, thought to be a fair one. Results summarized in the preceding paragraph, however, suggest that the assumption is not valid when rates of red blood cell formation and destruction are disturbed. Other exceptions also have been described. Two growing puppies fed radioiron were found to have approximately three and four times as much of the isotope in their tissues as in their blood.[8] They had been given the tagged iron during the course of twenty-seven days and had not been sacrificed until twelve days after the last dose. Utilization of the absorbed iron was so slow that a totally false impression would have been gained if the quantity which appeared in the blood as newly synthesized hemoglobin had been used as the only measure of absorption. Hahn and associates,[14] furthermore, found that fourteen days after radioiron as ferric ammonium citrate had been given intravenously to a normal dog the liver contained 82 per cent of the injected dose. These several considerations indicate clearly the need for an accurate definition of those circumstances in which iron utilization for hemoglobin synthesis is sufficiently complete that the radioactive technique can validly be used for measuring iron absorption.

For the sake of clarity, this paper will be divided into two parts. The first will present data on the utilization of injected radioiron; the second will relate this information to the radioactive iron method for studying absorption and will present additional pertinent data.

MATERIAL AND METHODS

The human subjects used were: (1) patients in the Barnes Hospital who volunteered as subjects and (2) healthy men, members of the house staff who had suffered no known blood loss and who had not recently served as donors for transfusions. The dogs were mongrels which had been kept in the animal quarters for at least a month, and usually much longer, before experimental observations were begun. During this time they were housed in individual cages, wormed, and fed Purina dog chow.

Hematologic techniques, methods for preparing samples and for measuring radioactivity,* were the same as those previously described.[12] The method of purifying the iron to free

*A metal Geiger counter tube with an aluminum window, supplied by the Physics Department of Washington University, has been used since 1945. This tube is less sensitive than the mica window counter which was used in the early part of this investigation but is more rugged.

it from radioactive cobalt and manganese, formed during the bombardment with deuterons in a cyclotron, has also been published in detail.[12] Radioactive ferrous ascorbate was prepared for intravenous injection from a stock radioactive ferric chloride solution as follows: Ferric hydroxide was precipitated by the addition of NaOH, the precipitate was spun down by centrifugation, and the supernatant liquid was discarded. Five parts of ascorbic acid to one part of iron were added to the precipitate in the centrifuge tube, and the mixture was stirred until the iron hydroxide dissolved. A few cubic centimeters of water were added, and the mixture was shaken vigorously for five minutes or until a purple solution was formed. This was poured into 20 volumes of acetone, the precipitated ferrous ascorbate was filtered off, and the salt was dried in a vacuum desiccator.[15] Solutions of ferrous ascorbate are unstable and are decomposed by autoclaving. The salt, therefore, was kept in the dry state until immediately before it was to be injected. It was then dissolved in sterile 5 per cent glucose in normal saline and a measured amount was injected. A small portion of each solution was kept for determination of the actual iron content and for preparation of a standard for the radioactivity determinations. The doses of ferrous ascorbate used were small (less than 0.5 mg. per kilogram of body weight) and caused no reactions. A few subjects noted tingling at the site of the injection and a few detected a metallic taste. Dogs were frequently given ferric chloride instead of ferrous ascorbate.

To determine the radioactive iron in urine, feces, and tissues, it was necessary to separate the iron from phosphates by ether extraction. The material was wet-digested with sulfuric and perchloric acids, and the digest was made alkaline with sodium hydroxide. The copious precipitate of phosphates was dissolved in 6 N HCl and extracted with ether that had previously been saturated with 6 N HCl, by shaking in a separatory funnel. Two extractions were usually sufficient. In the case of urine and tissues low in iron, 1 or 2 mg. of inert iron as $FeCl_3$ were added to the digest as a carrier. The iron was recovered from the ether layer by shaking with normal HCl, the solution was warmed to evaporate the ether, and the iron was precipitated as the hydroxide and electroplated and counted as previously described.[12]

In the calculation of the percentage of the dose which appeared in the peripheral blood, the blood volume was arbitrarily assumed to be 80 c.c. per kilogram. This assumption was probably no more inaccurate than would have been the estimation of red blood cell mass from determination of blood volume made by standard dye procedures. Correction was made for the iron removed in sampling.

I. Utilization of Intravenously Administered Radioactive Iron

A. *Healthy Human Subjects and Normal Dogs.*—Radioactive ferrous ascorbate was given intravenously in single doses which provided 9 to 18 mg. of the metal to four healthy young men, only one of whom had served as a donor within the previous year. The rate and completeness of iron utilization are illustrated in Fig. 1; hematologic data are recorded at the bottom of the chart. By the sixth day, 60 to 70 per cent of the injected iron had appeared in the peripheral blood; after this first rapid utilization, the amount increased slowly until 80 per cent or more was present at the end of seven to ten weeks. Positive errors in estimating blood volume probably account for the occasional values above 100 per cent. The prompt utilization during the first week is of considerable interest. If the injected iron had been added to the whole of the body's store of nonhemoglobin iron and had been used at the same rate as the stored iron was being mobilized, then it would have appeared much more slowly as newly synthesized hemoglobin. The result would seem to indicate that newly

injected iron is selectively used, initially at least, for new hemoglobin formation. Iron which has been stored for a longer period of time may not be so readily mobilized.

In normal dogs, parenterally administered radioiron was used less completely and somewhat more slowly than in the healthy human subjects. From 0.2 to 9.0 mg. of labeled iron, as ferric chloride or as ferrous ascorbate, were injected into thirteen animals. Results are charted in Fig. 2; the striated area delineates the extreme values obtained from the four healthy human subjects previously described and is included to make comparison easier. Even though

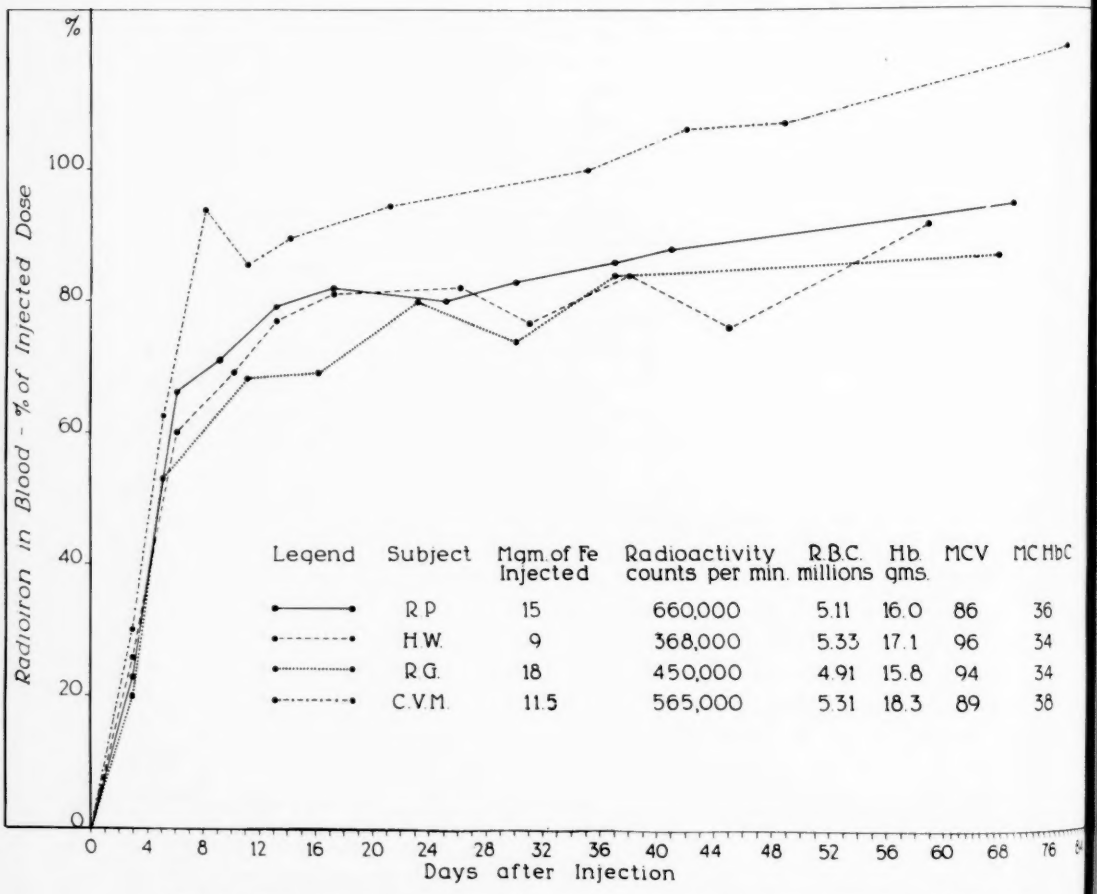
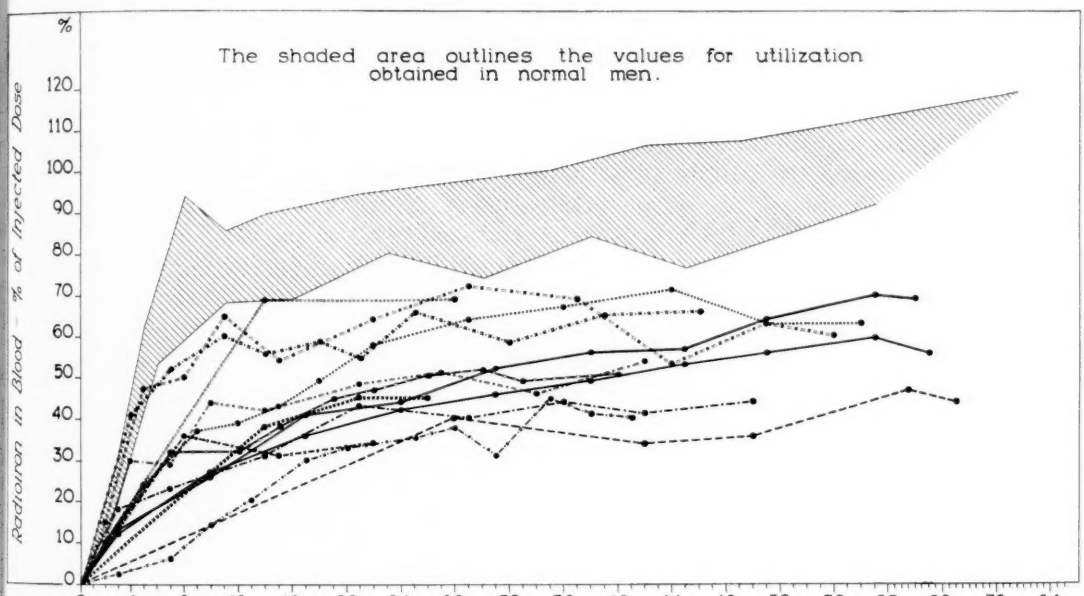

%
100
80
60
40
20
0
Radioiron in Blood - % of Injected Dose
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 68 76
Days after Injection
Legend Subject Mgm. of Fe Injected Radioactivity counts per min. R.B.C. millions Hb. gms. MCV MCHbC
R.P. 15 660,000 5.11 16.0 86 36
H.W. 9 368,000 5.33 17.1 96 34
R.G. 18 450,000 4.91 15.8 94 34
C.V.M. 11.5 565,000 5.31 18.3 89 38

Fig. 1.—The utilization of radioactive iron by normal men. The iron was given intravenously as ferrous ascorbate in the following doses: R. P., 0.19 mg. iron per kilogram of body weight; H. W., 0.12 mg. per kilogram; R. G., 0.21 mg. per kilogram; C. V. M., 0.13 mg. per kilogram.

observations were continued in several instances for sixty days or more, the total amount of the isotope which eventually appeared in the peripheral blood of these animals varied from 36 to 72 per cent of the amount injected, and in no instance was utilization as complete as in the human subjects. In only six of the dogs was 60 per cent or more of the injected dose found in the peripheral

blood at any one time. Completeness of utilization was not closely correlated with the size of the dose.* The animal (Dog 19) which received 9.0 mg. per kilogram body weight, for instance, used 45 per cent, while Dogs 55 and 58 given less than 1 mg. per kilogram also utilized only 47 and 45 per cent. On the other hand, the three dogs with the greatest utilization (Dogs 25, 26, 42) received 0.5 mg., 1.6 mg., and 5.0 mg. per kilogram, respectively. It should be emphasized, however, that even the larger amounts given were only tracer doses.



Legend	Dog	Wt. Kg.	Hb.	Form of Fe	Mg. Fe per Kg.	Radioactivity Counts per Min.
	19	17	17.6	$FeCl_3$	9.0	475,000
	25	21	16.3	$FeCl_3$	0.5	1,400,000
	26	16	15.4	$FeCl_3$	1.6	3,400,000
	42	15½	17.0	$FeCl_3$	5.0	1,900,000
	46	8	17.5	$FeCl_3$	0.5	580,000
	47	11	14.4	$FeCl_3$	0.5	730,000
	51	6	18.5	$FeCl_3$	1.3	1,120,000
	54	12	14.5	$FeCl_3$	0.5	144,000
	55	11	15.4	$FeCl_3$	0.5	144,000
	58	10½	16.2	Fe Ascorb.	0.7	600,000
	61	8½	15.4	Fe Ascorb.	1.3	580,000
	64	15	17.7	$FeCl_3$	1.0	1,800,000
	65	15½	16.5	$FeCl_3$	0.2	318,000

Fig. 2.—Appearance of radioactive iron in the blood of normal dogs after intravenous injection.

Attempts were made to find out what happens to the radioiron which does not appear in the peripheral blood as hemoglobin. Two animals (Dogs 58 and 51), in which the level of circulating radioactive iron had remained relatively constant for several weeks, were perfused for two hours under nembutal anesthesia with Locke's solution. The amount of the isotope which appeared in the

*Calculated in terms of the per cent of the administered dose, the *rate* of iron utilization was roughly proportional to the per kilogram size of the dose. With the larger amounts injected, however, more actual milligrams of iron were built into hemoglobin during the first week than with the smaller quantities.

perfusate and tissues was then determined. In Dog 58, the weights of the bone marrow, muscle, and skin were estimated, but in the other animal, a more complete sampling was obtained. All of the muscle was carefully cut from the body, ground, weighed, and an aliquot of the mixed tissue was taken for analysis. The whole skeleton was dry ashed, the ash was dissolved in 6 N HCl, and aliquots were taken for the radioiron determinations. The entire skin was removed and dry ashed; radioactivity was determined in HCl aliquots of the ash. In addition, all the radioiron excreted in the urine and feces of Dog 51 was measured for the whole fifty-day period; 8 per cent of the injected dose was recovered in the excreta. Half this amount was recovered in the urine within a few hours after injection of the isotope; however, after this brief period only small amounts appeared in the urine, and almost all of the additional excretion was by way of the intestinal tract. Results are recorded in Table I. The total amount of radioiron recovered in the tissues of these two dogs amounted to 17.1 and 41.6 per cent of the injected dose, but even so all of the iron could not be accounted for. If Dog 58 excreted as much of the dose as Dog 51, the total recovery would still be only 86 and 72 per cent, respectively. Hahn and associates[1] had a similar experience. The "lost iron" probably reflects the inadequacies of the method for extracting all of the iron from tissues and for measuring the isotope when amounts of radioactivity are small.

TABLE I. RADIOACTIVE IRON FOUND IN TISSUES OF TWO DOGS TWENTY-SIX AND FIFTY DAYS AFTER IRON HAD BEEN INJECTED INTRAVENOUSLY

	DOG 58	DOG 51
Weight (kg.)	10.5	6
Hematocrit	47	56
Hemoglobin (Gm.)	16.8	18.5
Radioiron injected (mg.)	7.3 (Ferrous ascorbate)	7.5 ($FeCl_3$)
Time sacrificed after I.V. iron (days)	26	50
Radioiron found in:	% of amount injected	
Blood and perfusion fluid	35.5	42.2
Bone marrow	10.3*	1.8
Liver	23.8	8.4
Spleen	1.0	0.3
Kidneys	1.1	0.6
Muscle	4.2*	3.3
Skin	1.2*	1.9
All other tissues	---	0.9
Excreted during experiment	---	8.3
Removed by sampling	0.9	4.2
Total radioiron accounted for	78.0	71.9

Although precise quantitative techniques were used throughout these studies, the precision of measurement of radioactivity is not high enough to justify carrying figures beyond the decimal point. It has, however, become a common practice in the literature, and we have consequently recorded our data to the first decimal place.

*Estimated weight of bone marrow was 300 Gm.; muscle, 3,000 Gm.; skin, 900 Gm.

In an additional attempt to recover all of the injected radioactive iron, three normal animals whose circulating hemoglobin level of radioiron had stabilized were bled several times a week for six to eight weeks. Two to three hundred cubic centimeters of blood were removed at each bleeding, and the total amount of radioactive iron withdrawn was measured (Table II). In two dogs, 16 and 28 per cent more of the injected isotope was removed in this manner than had

TABLE II. ATTEMPTS TO PULL RADIOIRON FROM TISSUES OF NORMAL DOGS BY SYSTEMATIC BLEEDINGS

DOG	WEIGHT (KG.)	ESTIMATED BLOOD VOLUME (C.C.)	HEMOGLOBIN (GM.)	HEMATOCRIT	RADIOIRON INJECTED (MG.)	RADIOIRON IN BLOOD VOLUME		RADIOIRON REMOVED BY BLEEDING			PULLED OUT OF TISSUES	EXCRETED	% IN BLOOD AT END OF EXPERIMENT	% OF DOSE ACCOUNTED FOR
						DAY	% OF DOSE	DAY	C.C. OF BLOOD	% OF DOSE				
61	8.6	690	17.7	51	11.6 (Ferrous ascorbate)	8	35.6	30 to 40	896	37.0				
						15	30.9	41 to 50	654	14.9				
						22	34.0	51 to 60	631	8.1				
						30	38.2	61 to 70	740	4.2				
								71 to 77	490	1.3				
								106	285	0.5				
								Total removed		66.0	27.8	6.0*		72.0
64	15	1,200	16.3	46	16.0 ($FeCl_3$)	26	50.0	0 to 40	52	1.7†				
						30	51.0	41 to 50	755	27.1				
						33	48.4	51 to 60	550	15.3				
						40	50.2	61 to 70	854	13.4				
						134	1.4	71 to 80	830	6.0				
								81 to 90	768	2.1				
								91 to 95	275	0.5				
								134	306	0.4				
								Total removed		66.5	16.3		1.4	67.9
65	15.5	1,240	16.5	47	3.2 ($FeCl_3$)	25	66.5	0 to 40	95	4.4†				
						32	58.0	41 to 50	553	24.7				
						39	64.6	51 to 60	757	18.3				
						46	66.5	61 to 70	585	10.2				
						134	5.4	71 to 80	900	6.6				
								81 to 85	550	3.1				
								134	298	1.6				
								Total removed		68.9	2.4		5.4	74.3

*Excreted in urine and stool in first three days.
†Removed in ten small samplings.

appeared at any one time previously in the circulating hemoglobin. These results also demonstrate that normal dogs do not regularly utilize injected radioactive iron completely for hemoglobin synthesis and at the same time show that the portion which had been stored is available for hemoglobin formation when the demand for iron becomes great enough. However, there still remained a fairly large amount (20 to 25 per cent) of the injected dose which was not accounted for, even if it be assumed that 10 per cent had been excreted during the period of observation.

B. *Iron-Deficient Patients and Dogs.*—Tracer doses of radioactive ferrous ascorbate were given intravenously to four patients with hypochromic microcytic anemia. As may be seen from Fig. 3, the rate of utilization was slightly more rapid than it had been in the normal controls. The amounts of iron

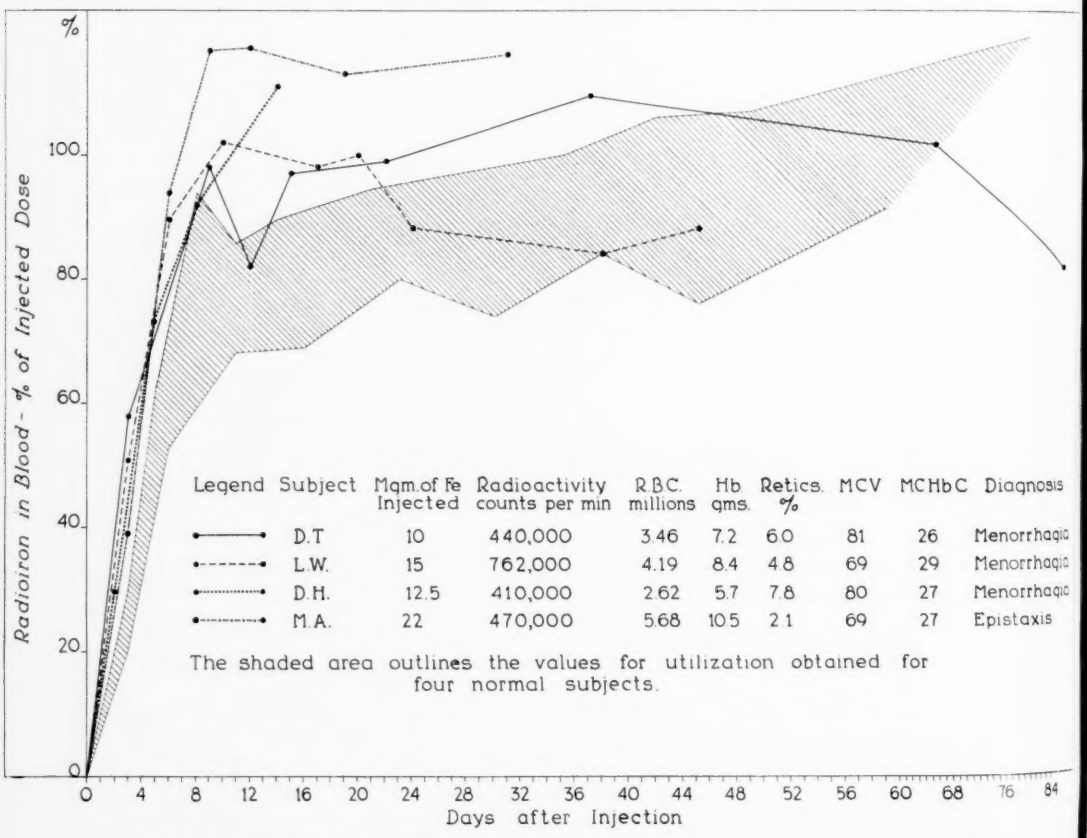
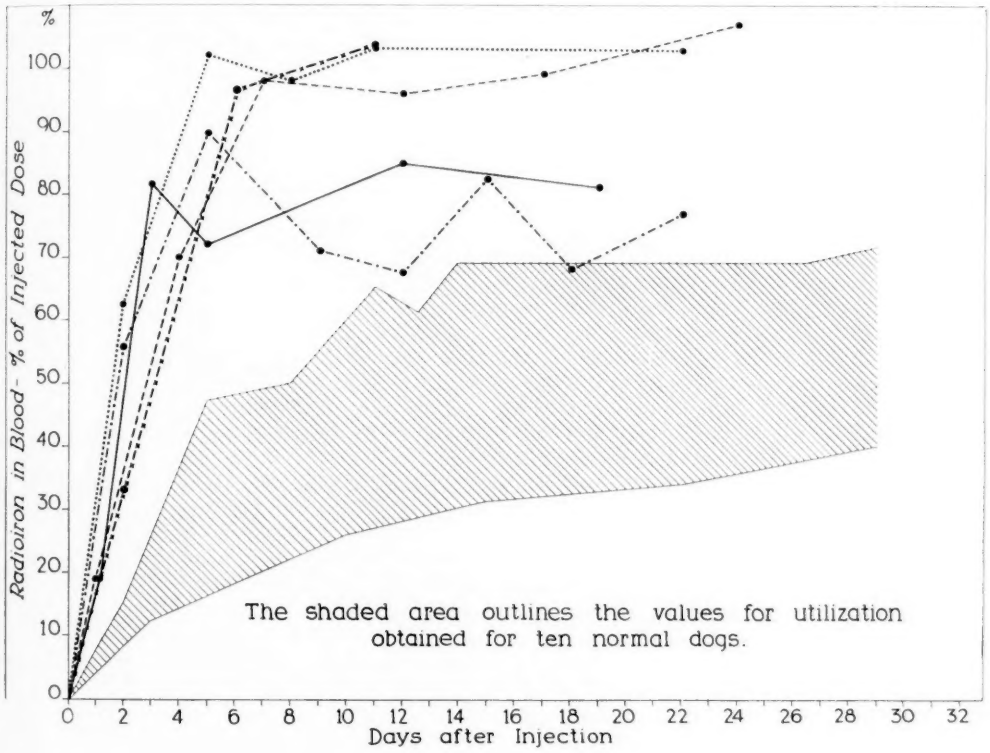

%
100
80
60
40
20
0
Radioiron in Blood - % of Injected Dose
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 68 76 84
Days after Injection
Legend Subject Mgm. of Fe Injected Radioactivity counts per min R.B.C. millions Hb gms. Retics. % MCV MCHbC Diagnosis
D.T 10 440,000 3.46 7.2 6.0 81 26 Menorrhagia
L.W. 15 762,000 4.19 8.4 4.8 69 29 Menorrhagia
D.H. 12.5 410,000 2.62 5.7 7.8 80 27 Menorrhagia
M.A. 22 470,000 5.68 10.5 2.1 69 27 Epistaxis
The shaded area outlines the values for utilization obtained for four normal subjects.

Fig. 3.—The utilization of radioactive iron by patients with hypochromic anemia. Iron given intravenously as ferrous ascorbate.

injected, the radioactivity, and the hematologic data for each individual are tabulated at the bottom of the illustration. In all four subjects, utilization of the injected iron was complete by the tenth day.

Iron-deficient dogs also promptly utilize 80 per cent or more of injected radioactive iron for hemoglobin synthesis (Fig. 4). Each of the five dogs was

prepared by withdrawing about 20 to 25 per cent of its blood volume two or three times a week for a period of two to five months; during this time the animals were given a diet of bread and milk so that the iron intake would be limited and the induced deficiency be more complete. A vitamin B supplement

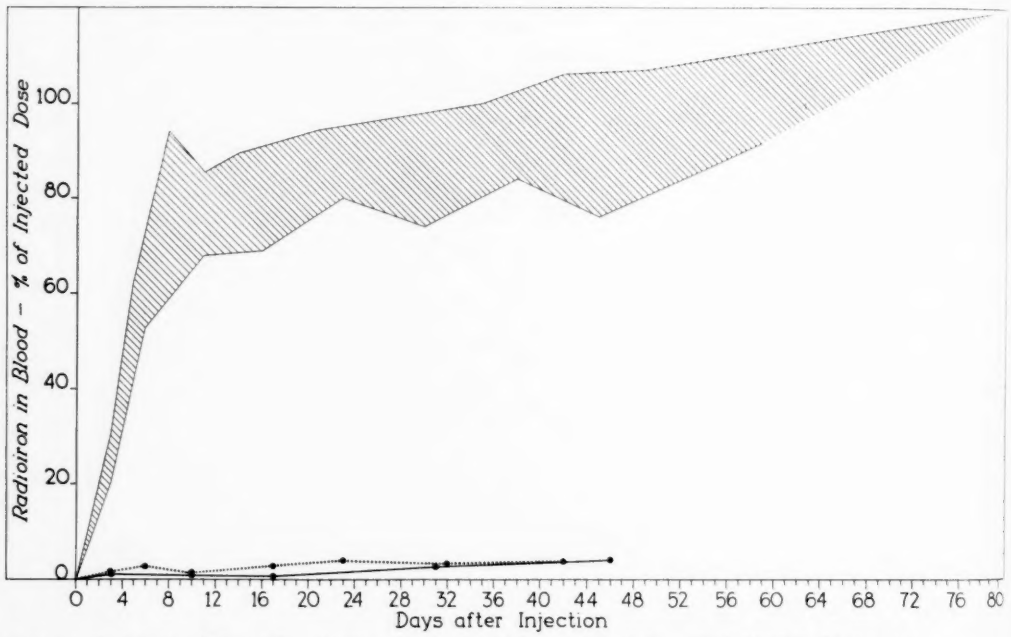


Legend	Dog	Wt. Kg.	Experiment	Hb	Mg. Fe per Kg.	Form of Fe Injected	Radioactivity Counts per Min.
	23	8.4	Anemic from hemorrhage	5.6	1.6	$FeCl_3$	1,800,000
	24	16.4	Anemic from hemorrhage	5.6	0.5	$FeCl_3$	1,130,000
	18	20.4	Anemic; massive dose of Fe; then R.I.	7.4	13.0	$Fe(OH)_3$; then $FeCl_3$	2,000,000
	21	13.2	Anemic, massive dose of Fe; then R.I.	5.7	26.0	$Fe(OH)_3$; then $FeCl_3$	2,100,000
	63	11.6	Anemic from hemorrhage	6.6	1.3	$FeCl_3$	204,000

Fig. 4.—The appearance of intravenously injected radioactive iron in the blood of five dogs which had been made anemic by chronic hemorrhage.

was regularly administered. The hemoglobin levels of the animals at the time radioactive iron was injected, with other pertinent data, are given at the bottom of the chart. Two of the animals (Dogs 18 and 21) were given 254 and 337 mg. of iron as colloidal ferric hydroxide intravenously shortly before the tracer

doses of radioiron were injected. These amounts provided enough iron to raise the hemoglobin from the existing levels of 5 to 7 Gm. to a value of 14 grams. It was thought that the administration of these relatively large amounts of inert metal might slow the utilization of the isotope, but the radioactive iron appeared as rapidly and almost as completely in the circulating hemoglobin as it had in the other three animals. From 70 to 90 per cent of the injected doses was utilized for hemoglobin formation.



Legend	Subject	Mgm. of Fe Injected	Radioactivity counts per min.	R.B.C. millions	Hb. gms.	Retics. %	MCV	MCHbC	Diagnosis
———	J.S.	18	500,000	4.15	13.3	0	94	34	Hypoplastic Anemia
·····•	E.H.	13	444,000	3.26	9.9	0.2	93	32	Hypoplastic Anemia

The shaded area outlines the values for utilization obtained for four normal subjects.

Fig. 5.—The appearance of radioactive iron in the blood of patients with hypoplastic anemia after intravenous injection of ferrous ascorbate.

C. *Patients With Hypoplastic Anemia.*—Radioactive ferrous ascorbate was injected intravenously into two patients with hypoplastic or refractory anemia. In both subjects, the blood platelet level was high enough to prevent any hemorrhagic manifestations. At the time of the injection, the erythrocyte counts had been raised to 4.15 and 3.26 million cells per cubic millimeter by previous transfusions. Additional blood had to be administered at intervals of two to three weeks during the course of the observations in order to prevent the red blood cell level from falling too low. The bone marrow showed very few erythroid elements, and reticulocytes were rare. In neither patient did as much as 4 per cent of the injected iron appear in the blood as hemoglobin during six weeks of observation (Fig. 5). This result is what would have been predicted.

If very little hemoglobin is being formed, there is little opportunity for the radioactive iron to be built into hemoglobin.

D. *Patients With Addisonian Pernicious Anemia.*—Study of the rate at which two patients with pernicious anemia in relapse utilized iron proved particularly interesting. For two weeks after the radioiron had been injected,

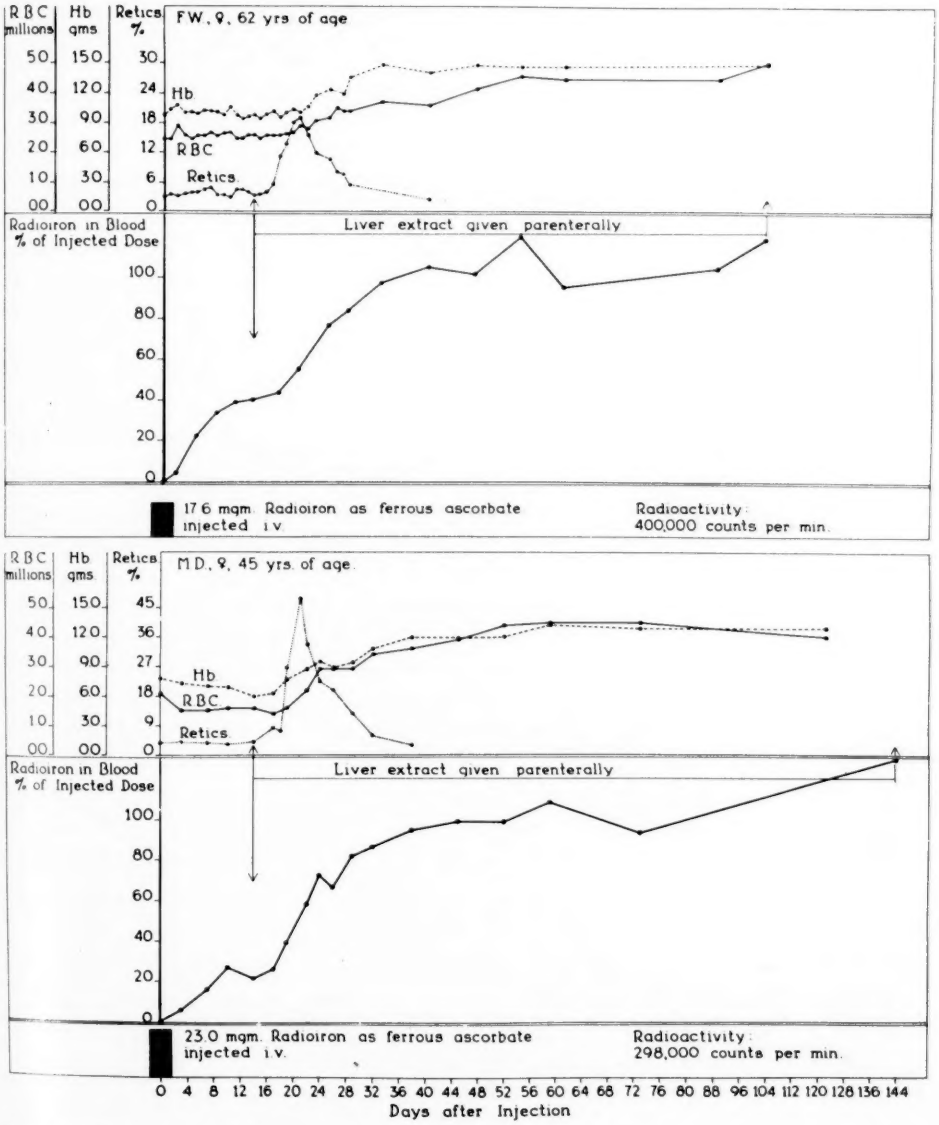
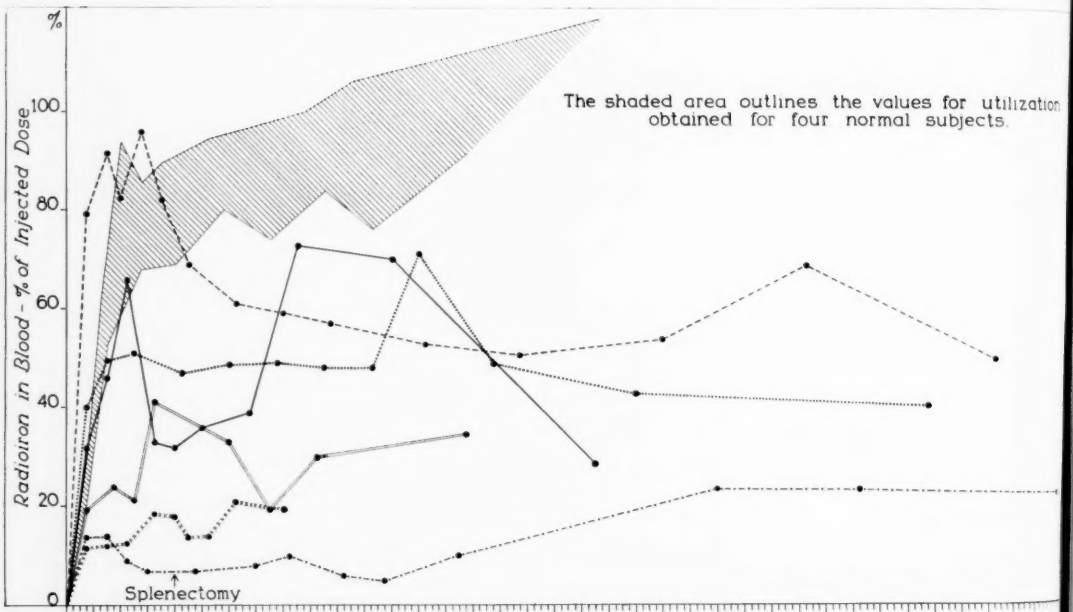

RBC millions
Hb gms
Retics %
F.W., ♀, 62 yrs. of age
Hb
RBC
Retics
Radioiron in Blood % of Injected Dose
Liver extract given parenterally
17.6 mgm. Radioiron as ferrous ascorbate injected i.v.
Radioactivity: 400,000 counts per min.
M.D., ♀, 45 yrs. of age
23.0 mgm. Radioiron as ferrous ascorbate injected i.v.
Radioactivity: 298,000 counts per min.
Days after Injection

Fig. 6.—The utilization of radioactive iron by two patients with pernicious anemia. Iron given intravenously as ferrous ascorbate.

therapy with liver extract was withheld, and the patients were fed a meat-free diet. The iron appeared rather slowly in the circulating hemoglobin and then tended to level off at about 20 and 40 per cent of the injected dose (Fig. 6).

Clinical circumstances demanded that specific therapy be started without additional delay, and the administration of liver extract was begun on the fifteenth day in each instance. Coincident with the reticulocyte rise, there was a sharp increase in the circulating radioiron, and within two to three weeks all of the isotope had been synthesized into hemoglobin. Observations were continued for three to five months; during this period the level of radioiron in the peripheral blood remained high. The initial slow rate of utilization was probably a


%
Radioiron in Blood - % of Injected Dose
100
80
60
40
20
0
The shaded area outlines the values for utilization obtained for four normal subjects.
Splenectomy
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68 72 76 80 84 88 92 96 100 108 116 124 132 140
Days after Injection

Legend	Subject	Mgm. of Fe Injected	Radioactivity counts per min.	R.B.C. millions	Hb gms.	Retics. %	MCV	MCHbC	Diagnosis
	R.P.	22	468,000	1.56	6.2	26.2	122	32	Acquired hemolytic anemia
	C.W.	13	534,000	3.49	13.4	15.9	112	34	Congenital hemolytic anemia
	B.W.	18	500,000	2.81	9.2	28.0	103	31	Sickle cell anemia
	T.S.	21	304,000	2.04	6.6	33.2	91	35	Sickle cell anemia
	M.W.	22	928,000	3.53	10.8	17.0	88	35	Sickle cell anemia
	G.P.	24	900,000	2.84	10.4	27.9	116	33	Sickle cell anemia

Fig. 7.—The appearance of radioactive iron in the blood of patients with hemolytic anemias after intravenous injection of ferrous ascorbate.

reflection of the relative erythroid maturation arrest which exists in patients with untreated pernicious anemia. As soon as red cell maturation was stimulated by liver extract, the rate at which new hemoglobin was delivered to the peripheral blood was accelerated, and the injected radioactive iron was utilized more rapidly.

E. *Hemolytic Anemias in Patients and Dogs.*—The effect of hemolytic anemias on the appearance of injected radioiron in the blood was studied in six patients: one with acquired hemolytic anemia, one with congenital hemolytic

anemia, and four with sickle-cell anemia.* Results are graphically recorded in Fig. 7. The initial rate of utilization was rapid in each instance but the rise usually ended abruptly, and in two of the patients the curves formed a plateau at less than 20 per cent of the dose. A value of over 90 per cent was reached in only one subject. It is suggested that because of the hemolytic process and the resultant rather short length of life of the red cells, many of the erythrocytes which contain radioactive iron in their hemoglobin are destroyed within a few days after their delivery to the peripheral blood. Variations in the rate of hemolysis probably account in large part for the different amounts of radioiron found in circulating hemoglobin at any one time. There were, furthermore, wide variations in the level of radioiron present in the same subject from week to week. These fluctuations are in decided contrast to the relatively smooth curves obtained in other patients and can probably also be explained on the basis of variations in the equilibrium between erythrocyte formation and destruction. A splenectomy was performed on the patient with acquired hemolytic anemia, and the excised spleen was found to contain 35 per cent of the radioiron that had been injected. After splenectomy, the patient's red blood cell count rose slowly to normal. With this improvement, the amount of circulating radioiron increased from 8.4 to 23.5 per cent of the injected dose. There are three probable reasons why a larger amount of the iron did not ultimately appear in the blood: (1) One-third of the injected dose had been removed in the spleen. (2) Some of the isotope may have been stored elsewhere. (3) Excretion of radioiron was probably significant, since it has been shown by Hahn and co-workers[16] that in dogs with hemolytic anemia induced by phenylhydrazine excretion is several times greater than normal.

Hemolytic anemia was induced in dogs by the oral administration of phenylhydrazine. All of the animals initially had hemoglobin levels above 15 Gm. and erythrocyte counts greater than 6.0 million cells. In Dogs 56, 60, and 59 (see Table III), the radioactive iron was given nineteen, twenty-nine and eighty-five days, respectively, after phenylhydrazine administration had been started, and the drug was continued for sixteen to twenty-eight additional days. The hemolytic anemia was maintained for two to four weeks after the radioiron had been injected, although the dose of phenylhydrazine was not regulated well

*Since this manuscript was submitted for publication, radioiron has been given intravenously to a seventh patient with hemolytic anemia. This patient was a white man, 58 years old, with an acquired hemolytic anemia; his red blood cell count was 1.3 million, and 36 per cent of his erythrocytes were reticulocytes. Fourteen milligrams of radioactive iron were given intravenously as ferrous ascorbate (0.19 mg. per kilogram of body weight). Three days later 20 per cent of the injected dose had appeared in the peripheral blood. Two days later the patient became acutely ill; he died on the seventh day after the injection. On the day of death the radioiron in the blood had dropped to 5.9 per cent. Assay of tissues obtained at necropsy gave the following results:

TISSUE	WEIGHT (GM.)	% OF INJECTED RADIOIRON
Spleen	630	19
Liver	2,540	40
Kidneys	430	1
Lungs	1,140	1
Bone marrow	3,360 (Estimated)	21
		Total found 82

Complete stool collections were made from the time the radioiron was injected until death. In spite of the intensity of the hemolytic process (17 Gm. of urobilinogen were excreted in the feces in three days), only 0.06 per cent of the injected radioiron appeared in the stools. Within four hours of the injection 1.3 per cent of the injected iron appeared in the urine. Of the injected dose, then, 1 per cent spilled over in the urine, 6 per cent was found in the red cells on the day of death, and 82 per cent was estimated to be in the tissues examined. Eleven per cent was not accounted for.

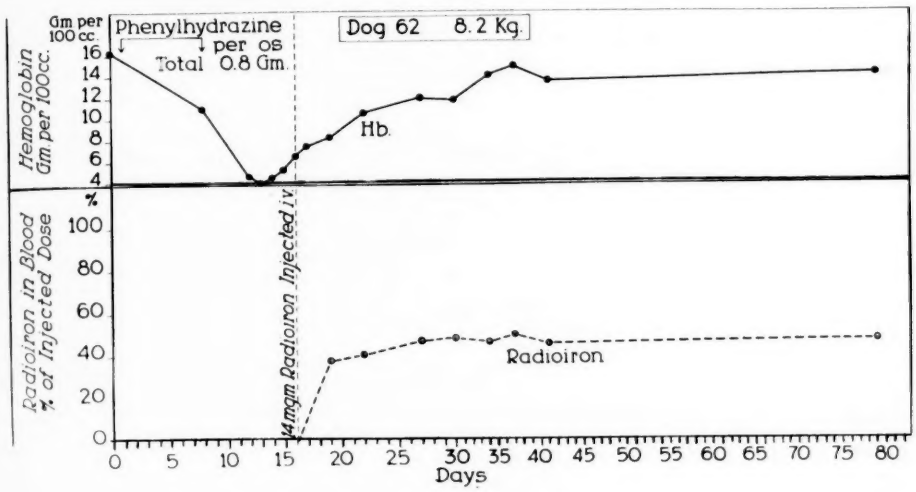
TABLE III. APPEARANCE OF RADIOIRON IN BLOOD OF DOGS WITH HEMOLYTIC ANEMIA INDUCED BY PHENYLHYDRAZINE

	DOG 29	DOG 56	DOG 59	DOG 60	DOG 62
Weight (kg.)	17	11.0	9.1	6.2	8.2
Initial hematologic data:					
R.B.C. (millions)	7.70	6.86	7.30	6.78	6.37
Hemoglobin (Gm./100 c.c.)	19.2	16.5	16.4	15.6	16.3
Phenylhydrazine (total days given)	39	35	113	49	7
Radioactive iron injected (I.V.):					
Days after start of phenylhydrazine	40	19	85	29	13
Milligrams per kilogram	0.5	1.0	1.1	1.7	1.4
Counts per minute in total dose	5,450,000	604,800	960,000	176,000	280,000
R.B.C. on day iron injected (millions)	2.95	2.55	2.21	1.39	1.83
Hemoglobin on day iron injected (Gm.)	9.4	6.5	4.9	3.3	6.6
Reticulocytes on day iron injected (%)	40.6	34.1	---	---	18.6
Time phenylhydrazine was discontinued:					
Days after injection of radioiron	---	16	28	20	---
Days before injection of radioiron	1	---	---	---	6
Maximum level of radioiron which appeared in blood (% of total dose)	66.5	46.9	30.5	52.6	49.4
R.B.C. at time of maximum level of radioiron	6.00	6.65	6.62	4.62	5.87
Hemoglobin at time of maximum level of radioiron	16.2	15.0	13.6	11.3	14.8
Radioiron removed by sampling and subsequent bleedings (% of dose)		---	---	51.1	53.5
Radioiron recovered in perfusate and blood, tissues, and in excreta collected in three weeks		70.1*			

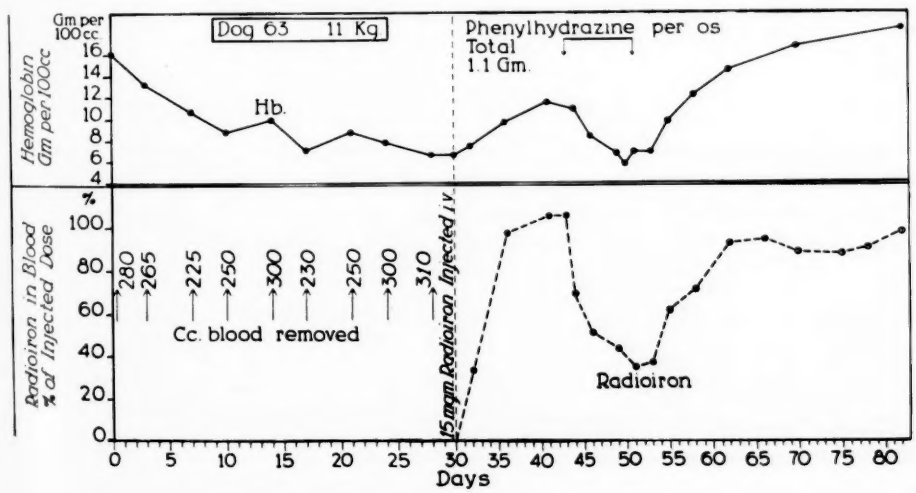
*Of this amount, 32.7 per cent was in blood and perfusate; 28.3 per cent was in the tissues; 6.5 per cent was recovered in urine and stools; and 2.6 per cent had been removed by sampling.

enough to keep the red blood cell and hemoglobin levels constant. The isotope appeared promptly in the peripheral blood, but in the first week only 13 to 22 per cent of the injected dose was demonstrated in the circulating hemoglobin. The levels stabilized at approximately these values until the phenylhydrazine was discontinued and the red cell count began to rise. The maximum amounts of radioiron which appeared in the blood of these three dogs, however, were 30.5, 46.9, and 52.6 per cent, respectively, in spite of the fact that the hemoglobin values rose sharply during the postphenylhydrazine period. It seemed possible that a greater proportion of the isotope might appear in circulating hemoglobin if the iron were injected after phenylhydrazine administration had been stopped, that is, at a time when the red blood cell count was definitely increasing. Accordingly, Dogs 29 and 62 were given the iron one and six days, respectively, after the last dose of phenylhydrazine (see Table III and Fig. 8). The maximum amounts of radioiron found in the blood of these two animals were 66.5 and 49.4 per cent, respectively, of the quantities injected; these values do not differ significantly from those obtained with the other three animals. To see whether the radioiron which had not appeared in the circulating hemoglobin was in a form which could be used for hemoglobin synthesis, two animals (Dogs 60 and 62) were subjected to massive bleedings for a period of weeks, and the total amount of isotope removed was determined. One of the animals had received the intravenous injection of iron before and the other after discontinuance of phenylhydrazine. In neither instance did the amount of iron

recovered by the bleedings differ significantly from that calculated as being present in the blood stream at the time the systematic venesections were begun (Table III). It seemed unlikely that all of the radioiron unaccounted for had been excreted, even though excretion in the presence of hemolysis may be greater



A.



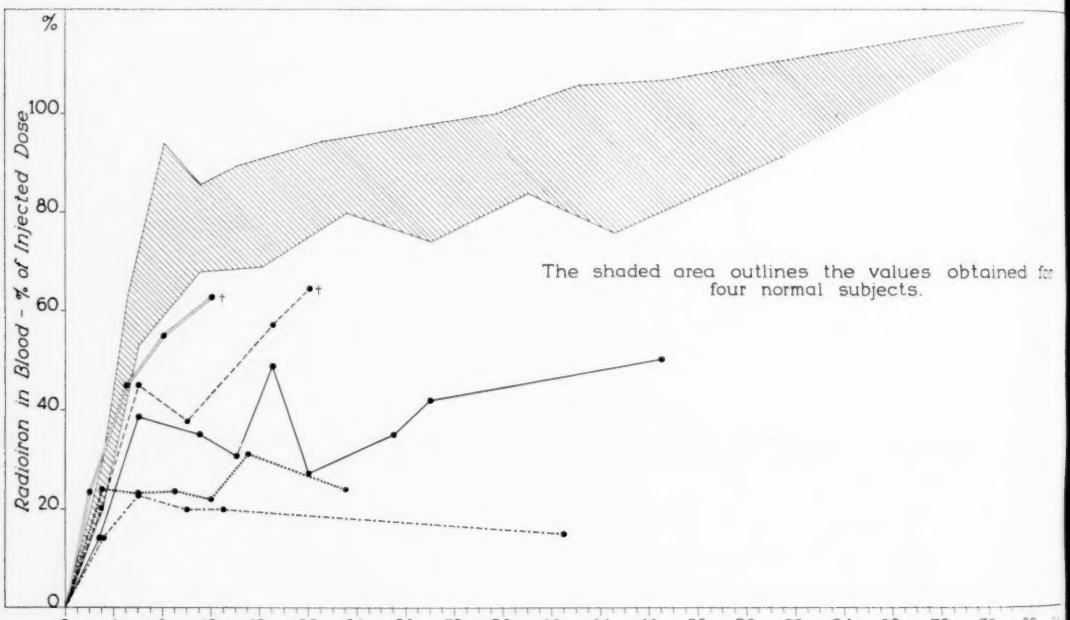
B.

Fig. 8.—*A*, The effect of a hemolytic anemia (phenylhydrazine) on the utilization of injected radioiron by a normal dog. *B*, The effect of a hemolytic anemia (phenylhydrazine) on the level of injected radioiron in the blood of an iron-deficient dog.

than normal.[16] To make certain, however, the excretion in urine and feces during three weeks of observation was measured in Dog 56, which had received phenylhydrazine for sixteen days following injection of the iron. Only 6.5 per cent of the dose could be accounted for in this manner. On the fiftieth day, the animal was anesthetized with nembutal, perfused, and sacrificed. The total

amount recovered in the perfusate, tissues, and excreta amounted to 70.1 per cent of the dose; 28.3 per cent was found in the tissues.

These results are not necessarily at variance with those reported by Cruz and associates.[17] The latter authors demonstrated that radioiron released from the hemoglobin of hemolyzed red cells could be reutilized for hemoglobin synthesis. Their published data, however, do not show what per cent of the injected dose was found in the peripheral blood at any one time.


%
Radioiron in Blood - % of Injected Dose
100
80
60
40
20
0
The shaded area outlines the values obtained for four normal subjects.
0 4 8 12 16 20 24 28 32 36 40 44 48 52 56 60 64 68 72 76 80
Days after Injection

Legend	Subject	Mgm. of Fe Injected	Radioactivity counts per min.	R.B.C. millions	Hb. gms.	Retics. %	MCV	MCHbC	Diagnosis
———	H.L.	13	240,000	5.00	14.9		86	35	Therapeutic malaria
- - - -	E.A.	22	468,000	3.40	9.8	4.8	88	33	Hodgkins' disease
———	B.L.	22	468,000	2.60	5.4	5.4	80	25	Carcinoma of kidney
- - - -	M.O.	2.5	128,000	3.70	9.0	1.2	84	29	Hodgkins' disease with gastrointestinal bleeding
———	H.C.	13	269,000	3.53	7.3	7.4	82	25	Hypernephroma

Fig. 9.—The appearance of radioactive iron in the blood of patients with fever after intravenous injection of ferrous ascorbate.

One other animal (Dog. 63) was partially depleted of its iron reserves. given radioiron intravenously, and permitted to utilize the injected iron completely for new hemoglobin before phenylhydrazine was administered (Fig. 8). With the development of the phenylhydrazine anemia, the circulating radioiron decreased to 35 per cent. When phenylhydrazine was discontinued and the animal was permitted to recover from its anemia, 98 per cent of the radioiron again appeared in the blood stream as circulating hemoglobin. This observation confirms Cruz's evidence that the radioiron released from liberated hemoglobin can be reutilized. When it is appraised in comparison with results ob-

tained on normal animals, however, it indicates that the normal dogs had such a plentiful supply of iron in their pools of iron available for hemoglobin synthesis[5, 6] that they used only a fraction of the tagged element.

F. *Patients With Fever or Chronic Disease.*—There is convincing evidence to indicate that iron utilization is impaired in the presence of fever or chronic disease.[3, 4, 18] The extent to which utilization is prevented, however, is not known. To obtain data in partial answer to this question, radioactive ferrous ascorbate was injected intravenously into five subjects: one with therapeutic malaria, two with Hodgkin's disease, and two with malignant tumors (Fig. 9). Three of the patients had low mean corpuscular hemoglobin concentrations; they were, then, iron deficient. In spite of this added stimulus to iron utilization, none of the subjects developed concentrations of circulating radioiron greater than 62 per cent of the dose, and in two the maximum amounts were less than 30 per cent.

II. Limitations of the Radioactive Iron Method for Measuring Iron Absorption From the Gastrointestinal Tract

From the observations presented in the preceding section, it is evident that not all of the radioactive iron introduced into the body necessarily finds its way to the peripheral blood as newly synthesized hemoglobin. Therefore, if that fraction of the oral dose of radioiron which appears in circulating hemoglobin is taken as the measure of absorption, the resultant values will frequently be low. Erroneous conclusions may, consequently, be drawn as to the ability of the animal organism to absorb iron under a variety of conditions. There is reason to believe that iron absorbed from the intestinal tract is used for hemoglobin synthesis in the same manner as is iron injected intravenously. If this is true, then, on the basis of the results described, one can make the following statements as to the adequacy of the radioactive iron technique for measuring iron absorption:

1. In subjects with uncomplicated iron deficiency, the method accurately measures absorption.

2. In normal adult men, the method also provides reasonably accurate values. The amount of radioactive iron which appears in circulating hemoglobin within six to nine days, however, may represent only 70 to 90 per cent of the dose, and further slight increases may occur during the subsequent three or four weeks. In healthy dogs, the circulating radioactive hemoglobin iron may be only 35 to 70 per cent of the iron retained.

3. In hypoplastic anemias and in untreated pernicious anemia, because of the slow rate of erythrocytogenesis, the amount of iron built into hemoglobin cannot be accepted as a measure of iron absorption.

4. In hemolytic anemias, the amount of radioactive iron built into hemoglobin and found in the peripheral blood at any one time may be anywhere from 10 to 90 per cent of that actually absorbed.

5. In patients with fever caused either by infection or other disorders, only 20 to 60 per cent of the absorbed radioiron may appear in circulating hemoglobin.

An excellent opportunity to test the validity of our concern about the method is provided by patients with pernicious anemia in whom erythrocytogenesis can be stimulated by injections of liver extract. Accordingly, the following observations were made: To E. G., a white man, 47 years of age, who had

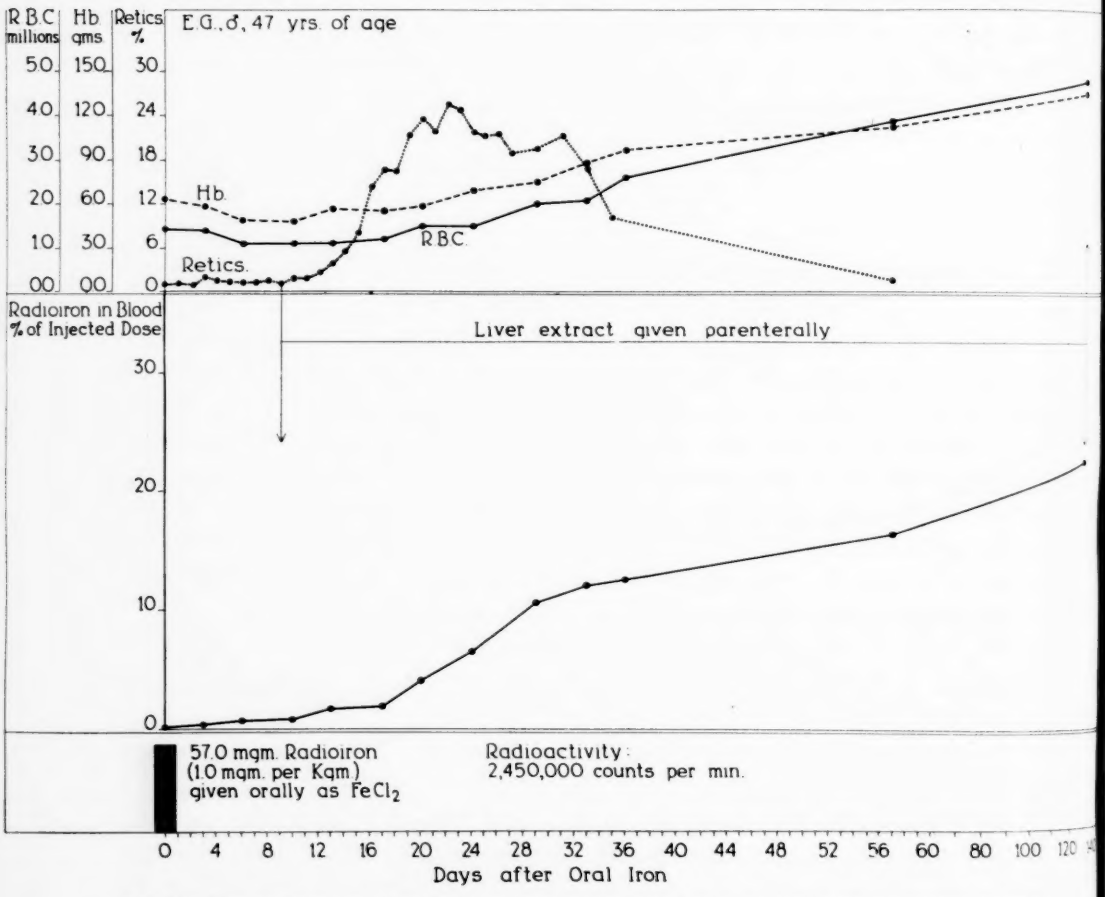


Fig. 10.—The appearance of radioactive iron in the blood of a patient with pernicious anemia after oral administration of ferrous chloride.

all of the classical clinical and hematologic manifestations of pernicious anemia in relapse, 1.0 mg. of radioactive iron per kilogram of body weight was given orally as ferrous chloride (Fig. 10). Only about 1 per cent of the dose appeared in the peripheral hemoglobin during the next nine days. This result was similar to that obtained by Ross and Chapin[11] and by Balfour and associates.[9] According to interpretations made by these investigators, this result indicated that the patient had absorbed a very small percentage of the oral dose. On the ninth day, intramuscular injections of liver extract were begun. There was the

usual reticulocytosis, clinical improvement, and subsequent rise in both red blood cell and hemoglobin levels. Coincident with the rise in hemoglobin, additional amounts of radioactive iron made their appearance in the blood stream; eventually more than 20 per cent of the administered oral dose was found in the circulating hemoglobin. Similar results on four additional patients will be reported later. This observation clearly illustrates the caution which must be used in interpreting results of studies made with the radioactive iron technique. The patient just described was certainly not iron deficient; he had enough iron stored at the time the isotope was given to raise his hemoglobin from 5 to more than 13 Gm. per 100 cubic centimeters.

DISCUSSION

While iron utilization can be studied more satisfactorily with radioactive iron than with any other technique previously available, results are definitely influenced by the rate of red blood cell formation, the rate of red cell destruction, and possibly by other disturbances of hemoglobin metabolism. The importance of the first of these influences is relatively obvious. When erythrocytogenesis is depressed, new hemoglobin is being synthesized so slowly that only small amounts of the radioiron can be used. In hemolytic anemias, however, new hemoglobin is being formed at a rapid rate, and large quantities of iron are being used for this purpose. One would naturally expect that radioactive iron given to such subjects would be incorporated quantitatively and promptly into hemoglobin. Yet, only a portion of the administered dose can be recovered in circulating hemoglobin at any one time. There are two possible reasons for this unexpected result. The amount of untagged or nonradioactive iron available in the storage depots, and constantly being released from hemoglobin liberated from hemolyzed red blood cells, may be so great that the body barely samples the available radioactive isotope in order to have enough of the metal for hemoglobin formation. In the second place, new red cells containing radioiron live a rather short time because of the hemolytic process, and the concentration of radioactivity isn't built up. All of the administered radioiron might eventually be incorporated into hemoglobin, but only a portion is found in the peripheral blood at any one time.

One of the most interesting results was the prompt and almost complete utilization of the injected isotope by healthy adult men. From 70 to 90 per cent of the dose was used for hemoglobin synthesis within the first six to nine days. The newly administered iron, therefore, was selectively used in preference to body iron already stored. If the new iron had been intimately mixed with stored iron, the isotope would have been sampled only, and long periods of time would have been required before an appreciable quantity would have been used for hemoglobin formation. This suggests that iron recently added to the body (and, probably, that released each day from destroyed erythrocytes) is retained in some form which is more easily mobilized for current metabolic needs than is iron which has been stored for a longer period of time. If one represented stored iron as being present in a bottle, then iron added to the stores would both float on top as does cream in a bottle of milk and be removed

first when the bone marrow or other tissues needed it. As a result of his studies of ferritin, an iron-protein complex asssociated with iron storage, Granick[19] postulates that there are three fractions of storage iron: *A*, monomolecularly dispersed iron compounds; *B*, colloidal iron compounds, namely, ferritin and nonerystallizable ferritin; and *C*, hemosiderin. Granick believes that *A* (ferrous and ferric compounds, probably in the form of their hydroxides) represents the metabolically active iron, which may be in equilibrium with *B* (ferritin). This impression is compatible with the suggestion just made. Similar ideas have recently been expressed by Ross[5] and by Greenberg and Wintrobe.[6] These workers postulate that there is a labile iron pool made up of recently absorbed iron and of iron recently liberated from destroyed red cells from which the body normally takes the amount needed each day. The concept is the same, but the term "labile iron pool" carries the unfortunate connotation that this amount may be held apart or separate from the remaining stores. It seems to us that the form of storage is simply one from which the iron is more readily mobilized.

This concept would explain many of the results obtained in this investigation. Normal dogs may use less of the injected radioiron for hemoglobin synthesis than do normal human subjects, because a greater amount of inert iron is available in the readily mobilizable form; the longer the isotope remains in tissues unutilized, the more inert iron becomes available from the hemoglobin liberated each day. This portion of the radioactive iron, therefore, becomes buried and is recalled only under conditions of great demand, as after extensive hemorrhage. The patient with acquired hemolytic anemia on whom a splenectomy was done recovered after the operation, and her hemoglobin level was restored to normal. Even with this rise, however, only small amounts of the isotope were extracted from her tissues for the hemoglobin regeneration, presumably because by this time the isotope had been mixed with so much inert iron equally available to the bone marrow that it was merely sampled rather than selectively used. On the other hand, one may assume that the patients with pernicious anemia used all of the isotope for hemoglobin synthesis following liver extract therapy because they were liberating hemoglobin from destroyed red cells at a rate too slow to "cover up" effectively the radioiron injected two weeks previously.

When comparison is made between the curves of appearance of radioactivity in the blood of subjects with hemolytic anemias and of patients with pernicious anemia before liver, a striking difference is noted. In the hemolytic anemias (Figs. 7 and 8), radioactivity increased very rapidly during the first few days and then rather promptly formed a plateau. In pernicious anemia, however, the rise was slow during the two weeks of observation prior to injections of liver extract (Fig. 6). This observation is interpreted as being additional presumptive evidence that hemolysis is not a prominent factor in the pathogenesis of the anemia in patients with pernicious anemia.

Finally, it should be emphasized that the radioactive iron technique when used on a living subject measures only the utilization of iron for hemoglobin synthesis; the amounts used for myohemoglobin and for formation of cellular enzymes cannot yet be followed.

SUMMARY AND CONCLUSIONS

1. The utilization of intravenously administered tracer doses of radioactive iron for hemoglobin synthesis has been studied in human subjects and in dogs, under normal conditions and in the presence of various types of anemia. It was found that:

(a) Utilization was prompt and complete in iron-deficient patients and dogs.

(b) Utilization by healthy adult men was somewhat slower but almost as complete. On the other hand, normal dogs used only 35 to 70 per cent of the injected isotope.

(c) Patients with hypoplastic anemia used less than 4 per cent of the tagged iron. In patients with pernicious anemia, utilization was likewise slow until after liver therapy was begun; it then was accelerated and became complete.

(d) In hemolytic anemias, the amounts of radioactive iron present in the peripheral blood at any one time varied widely from 10 to 90 per cent of the dose; rise in radioactivity was rapid during the first few days but usually stopped within a short time, so that the curves formed a plateau. However, in one dog which was made iron deficient before hemolysis was induced with phenylhydrazine, utilization was both prompt and complete during recovery from the hemolytic anemia.

(e) Utilization was slow and incomplete in patients with fevers caused either by infection or other associated disease. This result was obtained even when the subjects were also iron deficient.

2. To explain some of these results, it is postulated that iron recently made available for storage from absorption, parenteral administration, or from hemoglobin liberated from destroyed red blood cells is retained in a form which can be more readily mobilized for use than can iron which has been stored for longer periods of time. This recently stored iron seems to be used selectively for current metabolic needs.

3. Because injected radioactive iron is either not completely used for hemoglobin synthesis under a variety of conditions or does not all appear in the circulating hemoglobin at any one time, it is emphasized that the radioactive iron technique has limited value as a method of studying iron absorption from the gastrointestinal tract. An attempt has been made to define these limitations. The authors believe that the radioactive iron method for studying absorption is still the best available but desire to direct attention to its limitations so that results may not be interpreted erroneously. As an example, observations are presented to show that patients with untreated pernicious anemia may absorb considerable amounts of radioiron but that the isotope does not appear in the peripheral blood in large amounts until after erythrocyte maturation arrest has been relieved by administration of liver extract.

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SERUM LYSOLECITHIN IN RHEUMATOID ARTHRITIS, PREGNANCY, AND JAUNDICE AND IN NORMAL PERSONS

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THIS study was prompted by the observations of Bergenhem and Fahraeus[1] that lysolecithin was capable of causing spherocytosis and hemolysis of erythrocytes and that rapidly sedimenting blood after incubation shows normal erythrocyte sedimentation values. Since spherocytosis tends to prevent aggregation or rouleaux formation, it seemed desirable to determine to what extent the serum lysolecithin levels in rheumatoid arthritis were altered because there are increased sedimentation rates as well as mild anemic tendencies in this disease. Rouleaux formation of the erythrocytes is associated with increases in the rate of sedimentation.[2] Further, since pregnancy and jaundice are known to promote remission of symptoms in rheumatoid arthritis, the lysolecithin levels in these conditions were determined to see whether there was any similarity or difference in lysolecithin values.

A series of fifty serum lysolecithin determinations was made in patients with rheumatoid arthritis, pregnancy, jaundice, and in normal persons, as controls. At the same time determinations of erythrocyte sedimentation (Wintrobe method) and of packed-cell volume were made in an attempt to determine the possible relationship between the rate of erythrocyte sedimentation and serum lysolecithin content.

Method for Lysolecithin Determination.—The method used was a modification of that described by Singer[3] and was as follows: 10 c.c. of serum were treated with 100 c.c. of 95 per cent ethyl alcohol and filtered immediately; the filtrate was evaporated under a stream of air; the residue was then taken up in 5 c.c. of 95 per cent ethyl alcohol and diluted with 30 c.c. of ethyl ether and centrifuged for ten minutes at 20,000 r.p.m.; the supernatant fluid was decanted off and the precipitate dried in a vacuum jar. Then the precipitate was dissolved in 0.8 c.c. of physiologic saline solution, and dilutions were made in geometric series. A 2 per cent standard erythrocyte suspension was prepared using the blood of the same normal individual for all determinations. The standard cells were washed three times in physiologic saline solution and then suspended in saline solution. This cell suspension was drawn up to the 0.5 mark on standard leucocyte pipettes and diluted with the lysolecithin solutions to the 11 mark. The pipettes were then incubated at 37° C. for one hour, and cell counts were made in the usual manner in the hemocytometer. The percentage of hemolysis was determined by comparing the cell counts in each of the serial dilutions to those of the controls using physiologic saline solution instead of the lysolecithin solutions.

Summary of Results.—In all cases the erythrocytes appeared to become more or less spheroid in the presence of the lysolecithin as compared with the normal controls in physiologic saline solution (0.85 per cent NaCl). The amounts of hemolysis in ten patients with rheumatoid arthritis, ten icteric patients, twenty

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TABLE I. PER CENT HEMOLYSIS IN RHEUMATOID ARTHRITIS

PATIENT	C.E.S.R.* (MM.)	DILUTIONS (PER CENT) 1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
G. N.	46	93	40	0	0	0	0	0	0
P. A.	42	97	25	0	0	0	0	0	0
M. C. N.	22	95	68	16	0	0	0	0	0
G. N.	45	45	35	0	0	0	0	0	0
B. X.	3	86	80	35	0	0	0	0	0
B. E.	30	100	87	86	70	30	16	0	0
R. S.	35	100	100	0	0	0	0	0	0
H. S.	28	100	56	48	26	0	0	0	0
S. L.	12	65	30	10	0	0	0	0	0
B. T.	37	48	39	30	24	20	18	0	0
Average		83	56	23	12	5	3	0	0

*C.E.S.R., corrected erythrocyte sedimentation rate.

TABLE II. PER CENT HEMOLYSIS IN ICTERUS

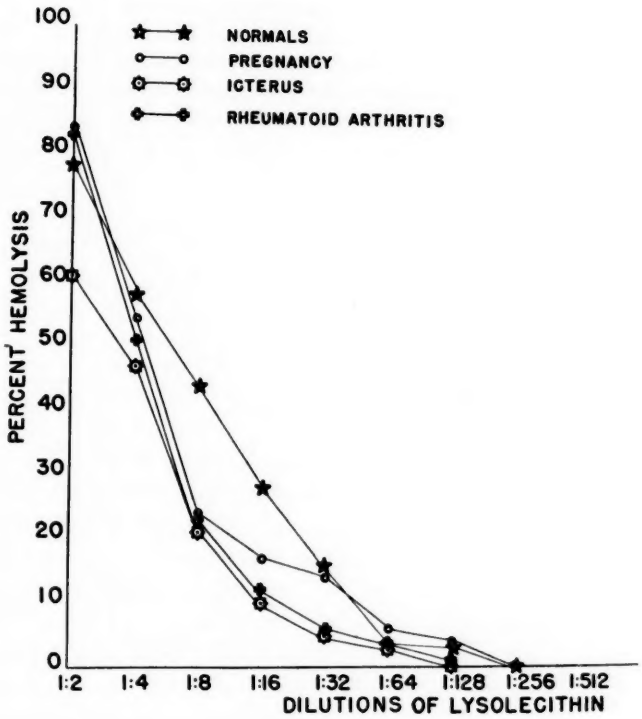
PATIENT	DIAGNOSIS	C.E.S.R.* (MM.)	I. I.†	DILUTIONS (PER CENT) 1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
S. W.	Hepatitis, acute	24	40	49	30	5	2	0	0	0	0
C. N.	Hemolitic anemia	6	20	66	38	20	8	0	0	0	0
S. S.	Hemolitic anemia	4	30	32	26	15	0	0	0	0	0
J. N.	Hepatitis, chronic	8	20	25	21	0	0	0	0	0	0
B. M.	Hepatitis, chronic	21	50	90	77	36	0	0	0	0	0
W. T.	Hepatitis, chronic	34	30	100	92	73	32	0	0	0	0
S. O.	Hepatitis, chronic	10	50	79	48	28	25	20	0	0	0
W. D.	Hepatitis, chronic	29	50	0	0	0	0	0	0	0	0
M. N.	Hepatitis, acute	14	80	67	36	36	36	26	26	0	0
H. N.	Hepatitis, chronic	34	190	97	88	0	0	0	0	0	0
Average				61	47	21	10	5	3	0	0

*C.E.S.R., corrected erythrocyte sedimentation rate.
†I.I., icteric index.

TABLE III. PER CENT HEMOLYSIS IN PREGNANCY

PATIENT	MONTH PREGNANT	C.E.S.R.* (MM.)	DILUTIONS (PER CENT) 1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512
M. N.	5	18	33	20	12	0	0	0	0	0	0
W. N.	7	22	28	23	14	14	14	17	17	0	0
B. N.	6	10	23	16	16	16	23	11	0	0	0
G. A.	6	24	77	59	4	0	0	0	0	0	0
R. Z.	7	35	86	48	50	66	40	0	0	0	0
H. S.	4	22	35	23	0	0	0	0	0	0	0
J. R.	7	25	37	42	22	22	18	15	0	0	0
M. L.	4	14	80	55	60	37	36	18	18	0	0
K. O.	7½	19	52	53	25	14	8	2	0	0	0
M. I.	7½	21	60	0	0	0	0	0	0	0	0
V. E.	4	12	60	50	12	0	0	0	0	0	0
W. N.	6	25	15	0	0	0	0	0	0	0	0
H. X.	6	10	38	0	0	0	0	0	0	0	0
S. T.	5	24	0	0	0	0	0	0	0	0	0
S. T.	4	29	42	15	0	0	0	0	0	0	0
K. Y.	5	34	11	16	16	0	0	0	0	0	0
F. N.	6	22	11	0	0	0	0	0	0	0	0
H. N.	6	38	30	30	0	0	0	0	0	0	0
G. H.	4	36	48	30	0	0	0	0	0	0	0
K. Y.	5	14	71	45	11	6	0	0	0	0	0
Average			84	53	24	17	14	6	4	0	0

*C.E.S.R., corrected erythrocyte sedimentation rate.


NORMALS
PREGNANCY
ICTERUS
RHEUMATOID ARTHRITIS
PERCENT HEMOLYSIS
100
90
80
70
60
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40
30
20
10
0
1:2
1:4
1:8
1:16
1:32
1:64
1:128
1:256
1:512
DILUTIONS OF LYSOLECITHIN

Fig. 1.

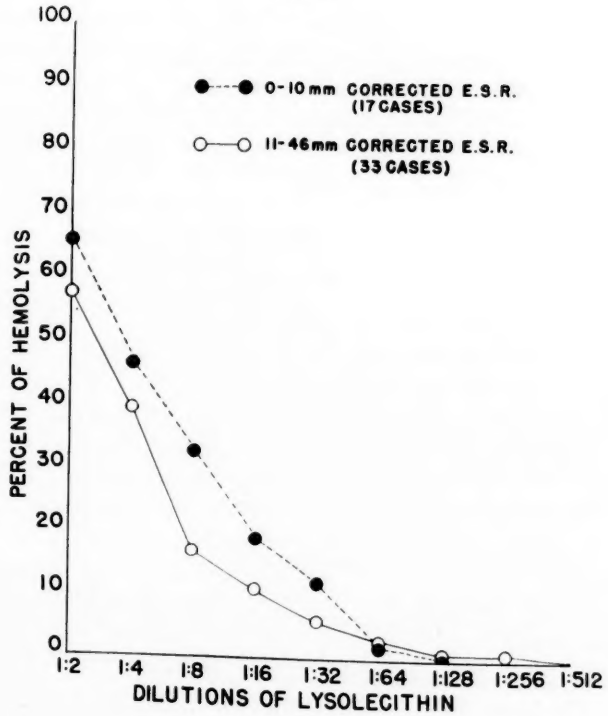
0-10 mm CORRECTED E.S.R.
(17 CASES)
11-46 mm CORRECTED E.S.R.
(33 CASES)
PERCENT OF HEMOLYSIS
100
90
80
70
60
50
40
30
20
10
0
1:2
1:4
1:8
1:16
1:32
1:64
1:128
1:256
1:512
DILUTIONS OF LYSOLECITHIN

Fig. 2.

TABLE IV. PER CENT HEMOLYSIS IN NORMAL PERSONS

PERSON	C.E.S.R.* (MM.)	DILUTIONS (PER CENT)							
		1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
K. L.	2	100	100	98	60	44	30	30	0
V. E.	0	75	67	56	28	0	0	0	0
C. G.	6	36	20	0	0	0	0	0	0
E. T.	2	97	74	51	43	34	0	0	0
D. M.	3	88	77	74	71	60	0	0	0
N. R.	6	100	17	10	0	0	0	0	0
C. E.	7	95	95	70	58	20	0	0	0
A. I.	4	87	38	36	20	0	0	0	0
H. N.	3	52	43	30	0	0	0	0	0
G. N.	0	50	44	15	0	0	0	0	0
Average		78	58	44	28	16	3	3	0

*C.E.S.R., corrected erythrocyte sedimentation rate.

pregnant patients, and ten normal persons are given in Tables I to IV, respectively. It is seen that in each table there was considerable variation from one patient to another as manifested by the percentage of hemolysis in the serial dilutions of lysolecithin. In other words, there was not a close adherence to a definite hemolysis curve for any of the conditions studied. Fig. 1 shows the average curves for each of the conditions studied.

The results are also grouped according to rate of erythrocyte sedimentation in Fig. 2 which shows the average curves for the seventeen cases with sedimentation rates of 0 to 10 mm. per hour and the thirty-three cases with sedimentation rates from 11 to 46 mm. per hour. It is seen that the characteristic average curves for each condition did not vary appreciably from one another. Furthermore, the lysolecithin content as manifested by hemolysis was not significantly different in those subjects with sedimentation rates below 10 mm. per hour and in those whose rates were over 10 mm. per hour. This was especially apparent when it is remembered that this method of determination is subject to about a 10 per cent error in measuring the percentage of hemolysis.

CONCLUSIONS

1. The average serum lysolecithin values, as measured by the amount of hemolysis of standard human erythrocytes, were found not to vary appreciably in rheumatoid arthritis, pregnancy, jaundice, and in normal persons as controls, but the individual variations were marked in these conditions.

2. There was a close correlation between the average lysolecithin values of persons with erythrocyte sedimentation rates below 10 mm. per hour and with those between 10 and 46 mm. per hour; therefore, the amount of lysolecithin did not appear to be related to the rate of erythrocyte sedimentation.

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THE RETAINED MEDIAN LETHAL DOSE, PERCENTAGE RETENTION, AND RESPIRATORY RESPONSE IN UNANESTHETIZED, INTACT RABBITS AND IN UNANESTHETIZED, TRACHEOTOMIZED DOGS AND RABBITS EXPOSED TO PHOSGENE BY THE DOSIMETRIC METHOD

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THE apparent species difference in susceptibility to phosgene previously reported on the basis of $L(Ct\dagger)_{50}$ data[1] was not resolved by determining the retained median lethal dose per kilogram of body weight in normal, unanesthetized dogs,[2] goats,[3] and monkeys.[4] However, only two of the factors, percentage retention and respiratory intake during exposure, which contribute to the variability of the $L(Ct)_{50}$ were eliminated by measuring the toxicity in terms of retained dose per kilogram of body weight.

Since the characteristic toxic effects of phosgene are exerted only on direct contact with the lower respiratory tract,[5] the proportion of retained agent which is absorbed by the respiratory epithelium above this locus of action may affect the apparent resistance of the animal to phosgene poisoning. Consequently, anatomic and physiologic dissimilarities in the upper respiratory tracts of different species may account, in part, for species variations in susceptibilty.

To evaluate the influence of absorption in the upper respiratory tract on the apparent sensitivity to phosgene, the retained median lethal dose was determined in unanesthetized dogs and rabbits in which the upper respiratory tract was by-passed by tracheotomy under local (procaine) anesthesia. Ease of handling and availability of dogs and rabbits led to the use of these animals as species representing high and low resistance[1] to phosgene, respectively. To check the marked susceptibility of rabbits previously reported on the basis of $L(Ct)_{50}$ data,[1] the retained median lethal dose was determined in a series of thirty-two intact, unanesthetized rabbits.

The dosimetric technique permitted the simultaneous determination of the percentage retention and the respiratory response during exposure.[6] Because some of these data suggested that depth of respiration influenced the percentage retention and the toxicity of phosgene, two groups of tracheotomized rabbits were studied, one breathing deeply with artificial respiration and the other breathing more shallowly without mechanical aid.

METHODS

All experimental exposures to phosgene were conducted at chamber concentrations of approximately 2.0 mg. per liter which, due to the dead space in the apparatus,[2] correspond to the following actual mean contact concentrations:

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†Ct = concentration × time.

1.48 mg. per liter for the thirty-five tracheotomized dogs; 1.25 mg. per liter for the thirty-two deep-breathing, intact rabbits; 1.52 mg. per liter for the thirty-two deep-breathing, tracheotomized rabbits; and 1.30 mg. per liter for the forty-three shallow-breathing, tracheotomized rabbits. The apparatus, procedures, and methods used for the dogs and rabbits in these experiments were similar to those previously employed in the dosimetric exposure of normal dogs[2] and monkeys,[4] respectively, except that for the tracheotomized animals no facepiece was required, since the distal end of each glass tracheal cannula consisted of the male half of a standard tapered joint for connection with the valve inlet of the apparatus.

The tracheotomies were performed under local anesthesia (1 c.c. of a 2 per cent solution of procaine hydrochloride for rabbits and 2 c.c. for dogs). After the exposure to phosgene, the cannula was removed carefully, and sulfathiazole crystals were applied in the cleansed wound which was left open for drainage. No deaths were observed in fifteen control rabbits and ten control dogs similarly cannulated and treated but not exposed to phosgene. The mean temperature and relative humidity during the exposures of the tracheotomized rabbits were 84.6° F. and 68.6 per cent, respectively; these corresponding data during exposures of the intact rabbits were 78.1° F. and 41.9 per cent, respectively, while those during the exposures of the dogs were 84.0° F. and 69.0 per cent, respectively.

In the intact normal rabbits, connection to the smaller glass valves and absorber was made by means of a vinylite nosepiece sealed to the male portion of a ground-glass joint. Great care was necessary to avoid obstructing the external nares. To test for possible leakage of the facepiece, a series of fifteen rabbits was prepared, as for dosimetric exposure, and permitted to inhale air from one spirometer and to exhale into another through the facepiece, valves, and absorber. In contrast to dogs, goats, and monkeys,[2-4] it was found that in rabbits, despite the most careful efforts to obtain an airtight seal, when the facepiece was applied to the snout with Ace elastic adhesive bandage, considerable leakage occurred (mean leakage, 56.4 per cent; standard deviation = ± 15.2 per cent; and standard error = 4.0 per cent). Therefore, the LD_{50} for rabbits, as determined with the leaking facepiece, was calculated and corrected by applying a factor of 1.77, based on the mean leakage observed in these tests. The standard error of the corrected LD_{50} was calculated as the sum of the standard error of the uncorrected LD_{50} plus the standard error of the mean leakage observed in the tests. It is recognized that the uncorrected standard error of the median lethal dose already includes the variation caused by leakage. However, to be conservative, the two standard errors were combined. Because of the leakage, the authors do not recommend that this method for dosimetrically exposing untracheotomized rabbits be used.

As a result of the marked reduction in tidal air during exposure of the intact rabbits, the small pressure changes transmitted to the valve system resulted in small fluttering movements of the flexible vinylite sheeting and produced very slight, slow respiratory deflections of the spirometer. To shorten the exposure times and to minimize rebreathing, artificial respiration was employed to increase tidal air and respiratory minute volumes.

RESULTS

The calculations and statistical treatment of these data were the same as described in a previous paper.[2] The complete experimental data for each animal are given in Tables I to IV.

Respiratory Response.—Respiratory inhibition in the normal, untracheotomized rabbits was so intense that, as previously stated, artificial respiration was employed to accelerate the exposures to phosgene. Therefore, no quantitative data were obtained on the spontaneous respiratory changs in these rabbits during exposure to phosgene.

None of the tracheotomized animals manifested any alteration of respiration during exposure, in contrast to the intact dogs,[2] goats,[3] monkeys,[4] and rabbits, which almost uniformly exhibited marked inhibition of respiration on exposure to similar concentrations of phosgene. The large, mean respiratory minute volume of 819 c.c. per kilogram per minute (Table V) and the relatively short exposures of the shallow-breathing, tracheotomized rabbits (Table III) indicate the absence of such inhibition in these animals.

The data in Table V permit a comparison between the average respiratory minute volume per kilogram during exposure of the normal and the tracheotomized dogs, which were 294 and 419 c.c. per kilogram per minute, respectively. The value (3.0) obtained by Student's t test reveals that the differences between these means is statistically significant (that is, $t \geqq 3$). Because dogs pant in response to increased environmental temperature and because there was a temperature difference of 7.3° F., comparison between the respiratory exchange in the two groups must be made cautiously. However, panting, nontracheotomized dogs also exhibit unequivocal respiratory inhibition exposure to phosgene. The absence of any respiratory inhibition in the tracheotomized dogs and the presence of the marked inhibition in the nontracheotomized animals clearly indicates that the inhibition results from the irritant action of phosgene on the upper respiratory tract.

Probably another consequence of the much higher temperatures during the exposures of the tracheotomized dogs is the fact that the average tidal air (Table VI) during exposure of this group is less than one-half that observed for the normal dogs.[2] The t value (9.8) reveals that this difference is highly significant.

The average tidal air of the shallow-breathing, tracheotomized rabbits, respiring without mechanical aid, was less than half that of the tracheotomized rabbits subjected to deep artificial respiration during exposure (Tables II and III). The t value (6.3) indicates that this difference is highly significant. The average respiratory minute volumes for the shallow-breathing and the deep-breathing tracheotomized rabbits were 819 and 408 c.c. per kilogram per minute, respectively. The t value (7.1) also indicates the marked significance of the difference between these two means. The influence of respiratory amplitude and rate of flow on toxicity and percentage retention of phosgene will be discussed.

Median Lethal Dose (LD_{50}).—The retained median lethal dose and the statistical range were calculated for each series by the method of Bliss.[7] After preliminary grouping of the animals to establish the provisional dosage-mortality regression line, each animal was considered a separate experiment. Agreement

TABLE I. PERTINENT DATA FOR THIRTY-TWO UNANESTHETIZED, INTACT, NORMAL RABBITS SUBJECTED TO DEEP ARTIFICIAL RESPIRATION DURING DOSIMETRIC EXPOSURE TO PHOSGENE AT CHAMBER CONCENTRATION* OF APPROXIMATELY 2 MG. PER LITER

RABBITS		PHOSGENE EXPOSURE					PHOSGENE RETENTION					RESPIRATION		
							DOSE RETAINED		PER CENT RETENTION					
SEX	WEIGHT (KG.)	VOLUME (LITERS)	CHAMBER CONCENTRATION* (MG./LITER)	TIME (MIN.)	CT† CORRECTED (γ-MIN./LITER)	TOTAL (MG.)	(MG.)	(MG./KG.)	OBSERVED	CORRECTION FACTOR	ACTUAL	NUMBER OF BREATHS	AVERAGE TIDAL AIR (C.C.)	SURVIVAL (HR.)
F	2.61	0.16	1.71	–	–	0.27	0.01+	0.01	5.1	–	–	–	–	Survived
F	3.07	0.16	1.72	–	–	0.28	0.03	0.01	10.9	–	–	–	–	Survived
F	2.79	0.18	1.71	–	–	0.31	0.05	0.02	16.2	–	–	–	–	Survived
M	2.87	0.19	1.71	–	–	0.33	0.07	0.02	20.7	–	–	–	–	Survived
F	2.50	0.19	1.72	–	–	0.33	0.07	0.03	21.2	–	–	–	–	Survived
F	3.64	0.22	1.71	–	–	0.38	0.19	0.05	50.4	–	–	–	–	Survived
F	3.18	0.33	1.60	–	–	0.53	0.17	0.05	32.4	–	–	–	–	Survived
F	2.79	0.23	1.73	–	–	0.40	0.18	0.06	44.6	–	–	–	–	Survived
F	3.95	0.38	1.81	–	–	0.68	0.29	0.07	42.0	–	–	–	–	48
F	2.61	0.20	1.82	–	–	0.36	0.17	0.07	47.8	–	–	–	–	48
F	3.35	0.31	1.79	–	–	0.56	0.27	0.08	48.9	–	–	–	–	Survived
F	3.41	0.44	1.79	–	–	0.79	0.28	0.08	35.9	–	–	–	–	Survived
F	2.76	0.24	1.69	–	–	0.41	0.23	0.08	55.3	–	–	–	–	Survived
M	2.73	0.24	1.69	–	–	0.41	0.23	0.09	56.9	–	–	–	–	Survived
F	3.64	0.28	1.69	–	–	0.48	0.32	0.09	65.6	–	–	–	–	Survived
M	2.87	0.40	1.60	–	–	0.64	0.25	0.09	39.0	–	–	–	–	48
F	2.99	0.64	1.70	–	–	1.09	0.26	0.09	23.5	–	–	–	–	Survived
F	2.76	0.32	1.78	–	–	0.57	0.28	0.10	49.1	–	–	–	–	Survived
F	3.52	0.45	1.69	–	–	0.76	0.38	0.11	49.6	–	–	–	–	Survived
F	2.44	0.48	1.75	–	–	0.85	0.39	0.16	46.3	–	–	–	–	Ca. 72
F	3.12	0.60	1.75	–	–	1.05	0.50	0.16	47.6	–	–	–	–	Ca. 24
F	2.86	0.63	1.77	–	–	1.12	0.44	0.16	39.7	–	–	–	–	Survived
F	2.58	0.96	1.70	–	–	1.63	0.46	0.18	28.3	–	–	–	–	Ca. 18
F	2.30	0.86	1.73	–	–	1.48	0.57	0.25	38.2	–	–	–	–	Ca. 24
F	2.81	0.96	1.70	–	–	1.63	0.75	0.27	45.8	–	–	–	–	Ca. 18
F	2.84	1.11	1.72	–	–	1.91	0.78	0.28	41.0	–	–	–	–	Ca. 20
F	3.35	1.02	1.79	–	–	1.83	0.99	0.30	54.2	–	–	–	–	Ca. 18
F	2.98	1.00	1.80	–	–	1.80	1.01	0.34	56.2	–	–	–	–	Ca. 5
F	2.47	0.86	1.72	–	–	1.48	0.86	0.35	58.4	–	–	–	–	Ca. 18
F	3.38	1.37	1.82	–	–	2.49	1.32	0.39	52.8	–	–	–	–	Ca. 18
F	2.47	1.01	1.79	–	–	1.81	1.00	0.40	55.0	–	–	–	–	Ca. 18
F	2.58	1.13	1.72	–	–	1.94	1.16	0.45	59.8	–	–	–	–	Ca. 18

*Chamber concentration × Correction factor = Mean contact concentration.
†Ct × Correction factor = Corrected Ct.

TABLE II. PERTINENT DATA FOR THIRTY-TWO UNANESTHETIZED, TRACHEOTOMIZED RABBITS SUBJECTED TO DEEP ARTIFICIAL RESPIRATION DURING DOSIMETRIC EXPOSURE TO PHOSGENE AT CHAMBER CONCENTRATION* OF APPROXIMATELY 2 MG. PER LITER

RABBITS TRACHEOTOMIZED		PHOSGENE EXPOSURE					PHOSGENE RETENTION					RESPIRATION		
							DOSE RETAINED		PER CENT RETENTION					
NO.	WEIGHT (KG.)	VOLUME (LITERS)	CHAMBER CONCENTRATION* (MG./LITER)	TIME (MIN.)	CT† CORRECTED (γ-MIN./LITER)	TOTAL (MG.)	(MG.)	(MG./KG.)	OBSERVED	CORRECTION FACTOR	ACTUAL	NUMBER OF BREATHS	AVERAGE TIDAL AIR (C.C.)	SURVIVAL (HR.)
1	2.27	0.416	1.965	0.28	423	0.817	0.270	0.119	32.9	77.0	42.7	25	16.65	Survived
2	1.73	0.172	2.065			0.355	0.211	0.122	59.5					Ca. 16
3	2.10	0.236	2.015	0.70	1,080	0.477	0.302	0.144	63.3	76.5	82.7	15	15.75	Survived
4	2.84	0.240	2.200	0.41	726	0.528	0.408	0.144	77.4	80.5	96.2	11	22.00	Survived
5	1.37	0.188	1.740	0.36	492	0.327	0.209	0.153	61.4	78.5	78.2	10	18.80	Survived
6	2.95	0.276	2.520	1.03	2,110	0.696	0.455	0.155	65.6	81.3	80.8	12	23.00	Survived
7	2.84	0.268	2.190	0.50	882	0.587	0.447	0.158	76.3	80.5	94.7	12	22.00	Ca. 16
8	2.73	0.260	2.240	0.30	552	0.582	0.437	0.160	75.0	82.5	90.9	9	26.00	Survived
9	2.36	0.468	1.900	0.51	771	0.889	0.385	0.163	43.8	79.6	55.0	23	20.35	Ca. 16
10	2.73	0.316	2.200	0.20	376	0.695	0.453	0.166	65.1	84.7	76.8	10	32.00	Survived
11	2.61	0.948	1.715	0.76	908	1.748	0.474	0.182	27.0	69.7	38.7	103	9.20	Survived
12	1.82	0.340	1.745	0.25	372	0.594	0.360	0.197	61.0	85.3	71.5	10	34.00	Survived
13	1.88	0.608	1.850	0.70	954	1.125	0.426	0.227	38.5	73.6	52.3	48	12.66	Survived
14	1.59	0.316	1.745	0.35	515	0.552	0.372	0.233	67.2	84.5	79.5	10	31.60	Ca. 16
15	2.04	0.470	1.810	0.23	336	0.850	0.330	0.255	38.8	80.8	48.1	21	22.39	Survived
16	1.90	0.320	2.055	1.60	2,514	0.658	0.495	0.261	75.4	76.5	98.5	20	16.00	Survived
17	2.64	0.492	2.250	0.53	960	1.107	0.690	0.261	62.3	80.5	77.4	22	22.00	Ca. 16
18	2.16	0.472	1.800	0.50	761	0.850	0.568	0.263	66.9	84.5	77.8	15	31.40	Survived
19	2.55	0.532	1.830	0.91		0.974	0.678	0.266	70.0					Survived
20	3.01	0.552	2.250	1.19	2,089	1.242	0.880	0.293	71.0	78.0	91.0	30	18.00	Ca. 16
21	2.64	0.520	2.250	0.41	775	1.170	0.783	0.296	66.8	84.0	79.5	18	29.00	Ca. 16
22	1.79	0.400	2.060	1.00	1,738	0.824	0.549	0.307	66.7	84.4	79.0	13	30.78	Ca. 16
23	1.88	0.600	1.675	0.34	437	1.050	0.624	0.332	59.0	76.8	76.7	37	16.22	Ca. 16
24	2.47	0.812	1.875	0.36	494	1.522	0.894	0.362	58.5	73.2	80.2	67	12.12	Ca. 16
25	1.93	0.596	1.915	0.44	750	1.141	0.729	0.378	64.0	89.0	71.9	49	12.18	Ca. 24
26	1.85	0.576	1.965	2.40	3,580	1.117	0.775	0.420	69.6	76.0	91.6	38	15.17	Ca. 16
27	1.79	0.508	2.060	1.00	1,694	1.047	0.822	0.459	78.6	82.1	95.7	20	25.04	Survived
28	1.67	0.528	2.050	1.70	1,851	1.082	0.770	0.461	70.8	81.8	86.5	25	21.15	Survived
29	1.67	0.536	2.060	1.10	1,804	1.104	0.774	0.464	70.0	79.6	88.0	26	20.60	Ca. 16
30	2.22	0.712	2.050	1.50	1,414	1.460	1.078	0.485	73.8	78.5	94.0	38	18.75	Ca. 16
31	2.39	0.888	2.090	1.40	2,413	1.856	1.362	0.570	73.3	82.5	88.9	34	26.10	Ca. 16
32	1.88	1.088	1.745	1.00	1,495	1.899	1.345	0.715	70.8	85.7	82.7	31	35.11	Ca. 16

*Chamber concentration × Correction factor = Mean contact concentration.
†Ct × Correction factor = Corrected Ct.

TABLE III. PERTINENT DATA FOR FORTY-THREE UNANESTHETIZED, TRACHEOTOMIZED RABBITS BREATHING WITHOUT ARTIFICIAL RESPIRATION DURING DOSIMETRIC EXPOSURE TO PHOSGENE AT CHAMBER CONCENTRATION* OF APPROXIMATELY 2 MG. PER LITER

RABBITS TRACHEOTOMIZED		PHOSGENE EXPOSURE					PHOSGENE RETENTION					RESPIRATION		
							DOSE RETAINED		PER CENT RETENTION					
NO.	WEIGHT (KG.)	VOLUME (LITERS)	CHAMBER CONCENTRATION* (MG./LITER)	TIME (MIN.)	CT† CORRECTED (γ-MIN./LITER)	TOTAL (MG.)	(MG.)	(MG./KG.)	OBSERVED	CORRECTION FACTOR	ACTUAL	NUMBER OF BREATHS	AVERAGE TIDAL AIR (C.C.)	SURVIVAL (HR.)
1	2.27	0.272	2.01	0.14		0.547	0.148	0.064	27.1					Survived
2	2.59	0.240	2.02	0.07		0.485	0.178	0.069	36.7					Survived
3	2.50	0.304	2.01	0.19		0.611	0.206	0.082	33.7					Survived
4	1.47	0.224	2.00	0.11		0.448	0.126	0.086	28.1					Survived
5	2.50	0.300	2.01	0.08		0.603	0.323	0.129	53.4					Survived
6	1.47	0.320	2.01	0.23		0.643	0.206	0.140	32.1					Survived
7	2.20	1.264	1.90	0.75	955	2.402	0.372	0.169	15.5	67.0	23.1	174	7.26	Survived
8	2.05	0.764	1.70	0.54	625	1.296	0.376	0.184	29.1	68.3	42.6	94	8.12	Survived
9	1.93	0.912	1.73	0.46	536	1.579	0.371	0.192	23.5	67.4	34.9	122	7.48	Survived
10	2.95	0.932	1.96	0.37		1.827	0.584	0.198	32.0					Survived
11	2.73	0.880	1.96	0.41		1.720	0.550	0.201	32.0					Survived
12	3.07	1.612	1.76	0.70	852	2.836	0.626	0.204	22.1	69.2	31.9	184	8.76	Ca. 16
13	2.13	1.568	1.70	0.73	889	2.658	0.558	0.262	21.0	71.8	29.2	144	10.90	Survived
14	2.95	0.924	2.02	0.43		1.867	0.797	0.270	42.7					Survived
15	2.05	1.496	1.68	1.14	1,249	2.511	0.561	0.274	22.3	65.2	34.2	242	6.18	Survived
16	2.02	1.468	1.74	0.87	1,019	2.527	0.557	0.276	22.1	67.5	32.6	194	7.56	Survived
17	2.24	1.276	1.90	0.57	724	2.424	0.668	0.278	27.6	66.9	41.2	177	7.21	Ca. 16
18	2.33	1.676	1.71	0.74	871	2.863	0.673	0.289	23.5	68.8	34.2	197	8.51	Ca. 16

19	1.82	0.580	1.97	0.48		1.142	0.527	0.290	46.2					Survived
20	2.13	1.516	1.74	0.68	818	2.536	0.640	0.300	25.3	69.3	36.4	172	8.82	Ca. 75
21	1.73	1.252	1.72	0.61	707	2.155	0.525	0.303	24.4	67.4	36.2	167	7.49	Survived
22	2.13	1.576	1.68	0.80	897	2.646	0.686	0.322	25.9	66.7	38.8	224	7.03	Survived
23	2.05	0.652	1.99	0.47	730	1.293	0.745	0.363	57.6	78.2	73.7	36	18.11	Ca. 90
24	1.99	1.664	1.85	0.88	1,087	3.080	0.769	0.386	25.0	66.8	37.4	234	7.11	Ca. 16
25	1.62	0.876	1.75	0.86	1,016	1.530	0.642	0.396	42.0	67.7	62.0	114	7.68	Survived
26	2.27	2.144	1.97	1.43	1,939	4.223	0.903	0.398	21.4	68.8	30.0	253	8.48	Ca. 16
27	2.45	1.412	1.90	0.64	893	2.683	1.061	0.433	39.6	73.4	53.8	114	12.39	Ca. 16
28	2.27	2.172	1.95	1.21	1,634	4.223	0.998	0.440	23.6	69.4	34.1	244	8.90	Ca. 85
29	1.34	0.940	1.76	1.11	1,270	1.645	0.605	0.452	36.8	65.2	65.5	152	6.18	Survived
30	2.36	1.972	1.90	0.95	1,253	3.737	1.132	0.480	30.3	69.6	43.3	227	8.69	Survived
31	1.96	1.276	1.87	1.41	1,607	2.382	0.986	0.503	41.4	65.6	63.0	200	6.38	Ca. 16
32	2.24	1.292	1.90	1.21	1,679	2.453	1.195	0.533	48.7	73.0	66.7	108	11.96	Ca. 16
33	2.36	1.836	1.84	1.14	1,463	3.378	1.278	0.542	37.9	69.8	54.3	200	9.18	Ca. 16
34	2.30	2.524	1.79	0.80	947	3.602	1.272	0.553	35.3	66.3	53.3	295	6.84	Ca. 16
35	2.02	1.328	1.90	2.33	3,882	2.508	1.123	0.556	44.2	87.7	50.4	31	42.82	Ca. 16
36	2 50	2.092	1.82	1.31	1,907	3.790	1.435	0.574	37.9	80.2	47.2	188	11.13	Ca. 16
37	1.73	1.148	1.89	1.27	1,657	2.166	1.041	0.602	48.1	69.0	69.5	133	8.63	Survived
38	1.93	1.688	1.79	0.75	858	3.014	1.228	0.636	40.7	67.1	60.6	232	7.27	Ca. 16
39	2.41	2.316	1.94	1.67	2,223	4.491	1.491	0.656	33.2	68.6	48.3	280	8.27	Ca. 16
40	1.93	1 264	1.89	0.92	1,168	2.390	1.287	0.666	53.9	67.2	80.2	172	7.33	Ca. 20
41	1.73	0.956	1.90	1.09	1,383	1.818	0.308	0.755	16.9	66.8	25.4	137	7.05	Ca. 16
42	1.90	1.464	1.93	2.54	2,850	2.824	1.567	0.825	55.5	57.6	99.2	198	2.54	Ca. 16
43	2.75	2.568	1.98	1.20	1,990	5.083	2.283	0.830	45.1	83.7	54.8	222	11.56	Ca. 16

*Chamber concentration × Correction factor = Mean contact concentration.
†Ct × Correction factor = Corrected Ct.

TABLE IV. PERTINENT DATA FOR THIRTY-FIVE UNANESTHETIZED, TRACHEOTOMIZED DOGS EXPOSED BY DOSIMETRIC METHOD TO PHOSGENE AT CHAMBER CONCENTRATION* OF APPROXIMATELY 2 MG. PER LITER

DOGS TRACHEOTOMIZED		PHOSGENE EXPOSURE					PHOSGENE RETENTION					RESPIRATION		
							DOSE RETAINED		PER CENT RETENTION					
SEX	WEIGHT (KG.)	VOLUME (LITERS)	CHAMBER CONCENTRATION* (MG./LITER)	TIME (MIN.)	CT† CORRECTED (γ-MIN./LITER)	TOTAL (MG.)	(MG.)	(MG./KG.)	OBSERVED	CORRECTION FACTOR	ACTUAL	NUMBER OF BREATHS	AVERAGE TIDAL AIR (C.C.)	SURVIVAL (HR.)
M	10.45	10.628	1.96	3.13	4,250	20.83	6.43	0.615	30.9	69.2	44.6	286	37.2	Survived
F	9.55	10.565	1.99	2.49	3,430	20.98	5.93	0.621	28.3	69.2	40.8	283	37.3	Survived
M	8.18	10.496	2.06	3.76	5,382	21.62	5.72	0.699	26.4	69.5	38.0	271	38.7	Survived
F	6.36	8.664	1.89	2.63	3,395	16.34	4.49	0.706	27.5	68.3	40.2	Ca. 250	34.6	Ca. 87
M	14.13	13.160	1.90	3.59	5,061	25.00	10.25	0.727	41.0	74.2	55.3	234	56.2	Survived
F	10.23	10.560	1.93	2.65	5,380	20.38	8.03	0.785	39.4	69.3	56.8	279	37.8	Survived
M	9.10	9.440	1.90	1.30	1,706	17.94	7.94	0.794	44.3	69.1	64.0	256	36 9	Survived
M	10.91	10.684	2.11	3.87	5,608	22.56	8.91	0.816	39.5	68.7	57.5	301	35.5	Survived
F	10.90	16.390	1.98	8.42	11,300	32.37	9.12	0.836	28.2	67.8	41.6	495	33.1	Ca. 22
F	10.91	11.176	1.98	4.12	5,840	22.07	8.27	0.849	42.0	71.6	58.7	246	45.4	Survived
F	10.45	10.432	1.91	2.91	3,926	19.92	9.02	0.863	45.5	70.4	64.7	252	41.4	48
F	17.50	16.612	1.91	4.42	6,321	31.73	15.43	0.882	48.6	74.9	64.9	280	59.3	Ca. 90
F	5.91	8.092	1.94	3.52	4,864	15.70	5.55	0.939	35.5	71.2	49 8	184	44.0	Survived
M	10.68	13.860	2.05	3.93	5,958	28.42	10.17	0.952	35.8	73.9	48.5	253	54.8	Survived
M	6.36	9.036	1.96	1.93	2,658	17.81	6.16	0.968	34.6	70.2	49.3	221	40.8	Survived
M	9.80	8.660	2.05	3.84	5,980	17.75	10.20	0.985	57.5	76.0	75.7	133	65.2	Survived
F	11.82	13.452	1.95	1.30	1,880	26.23	11.83	1.001	45.2	74.2	60.9	240	56.0	Survived
M	7.27	7.332	1.91	2.27	3,088	14.00	7.30	1.004	52.2	71.2	73.3	166	44 1	Survived
F	11.40	14.960	2.05	2.89	4,332	30.67	11.47	1.005	37.4	73.1	51 2	290	51.5	Survived
M	13.60	13.740	2.00	3.50	5,250	27.48	13.68	1.005	49.8	75.0	66.5	227	60.5	Survived
M	15.91	17.092	2.04	2.64	4,242	34.87	16.37	1.029	47.0	78.8	59.7	211	80.9	27
M	7.27	7.356	1.96	0.97	1,393	14.41	7.51	1.033	52.1	73.2	71.3	142	51.8	Survived
F	12.10	17.310	2.05	4.60	6,450	35.49	12.64	1.045	35.6	68.4	52.0	496	34.9	Survived
M	13.60	16.950	1.95	8.24	11,955	33.08	14.38	1.057	43.5	74.4	58.5	296	57.3	Survived
M	13.40	11.710	2 00	4.11	6,205	23.42	13.87	1.070	59.2	76.5	77.4	174	67.3	Ca 40
F	13.60	25.278	2.00	4.60		50.56	15.46	1.136	30.5					Survived
F	6.82	7.496	2.06	1.82	2,700	15.44	7.89	1.157	51.1	72.0	71.0	159	47.0	Survived
F	5.45	6.408	1.92	2.86	3,659	12.28	4.63	1.217	54.0	66.6	81.0	214	29.9	Ca. 36
M	6.36	9.236	1.93	1.84	2,549	17.83	7.93	1.246	44.5	71.8	62.0	198	46.6	Ca. 24
F	5.68	6.272	2.00	3.10	4,546	12.55	7.40	1.302	59.0	73.3	80.5	120	52.3	Survived
F	7 27	7.872	1.98	4.18	6,374	15.55	10.65	1.465	68.5	77.0	88.9	113	69.7	Ca. 24
F	10.91	17 688	1.92	5.43	7,540	33.84	16.09	1.473	47.5	72 3	65.7	365	48.4	Ca. 16
F	6.36	9.768	2.03	4.20	6,700	19.86	10.16	1.597	51.2	78.5	65.2	182	53.7	Ca. 16
M	10.91	16.704	1.97	8.17	13,170	32.83	19.63	1.799	59.9	81.8	73.2	240	69.6	Ca. 16
M	9.30	15.040	2.04	2.05	3,145	30.61	20.86	2.242	68.1	75.2	90.5	247	60.8	Ca. 24

*Chamber concentration × Correction factor = Mean contact concentration.
†Ct × Correction factor = Corrected Ct.

TABLE V. AVERAGE RESPIRATORY MINUTE VOLUME PER KILOGRAM BODY WEIGHT IN NORMAL AND TRACHEOTOMIZED DOGS AND IN SHALLOW- AND DEEP-BREATHING, TRACHEOTOMIZED RABBITS DURING EXPOSURE TO PHOSGENE CONCENTRATIONS OF 1.30 TO 1.52 PER LITER

EXPERIMENTAL ANIMALS	NUMBER OF ANIMALS	RESPIRATORY MINUTE VOLUME PER KILOGRAM DURING EXPOSURE		
		MEAN (C.C./MIN./KG.)	STANDARD DEVIATION (C.C./MIN./KG.)	STANDARD ERROR (C.C./MIN./KG.)
Normal dogs	30	294	118	21
Tracheotomized dogs	35	419	217	37
Shallow-breathing tracheotomized rabbits	43	819	266	41
Deep-breathing tracheotomized rabbits	32	408	234	41

TABLE VI. AVERAGE TIDAL AIR VOLUME PER KILOGRAM BODY WEIGHT IN NORMAL AND TRACHEOTOMIZED DOGS AND IN DEEP- AND SHALLOW-BREATHING RABBITS DURING EXPOSURE TO PHOSGENE CONCENTRATIONS OF 1.30 TO 1.52 MG. PER LITER

EXPERIMENTAL ANIMALS	NUMBER OF ANIMALS	AVERAGE TIDAL AIR VOLUME PER KILOGRAM BODY WEIGHT		
		MEAN (C.C./KG.)	STANDARD DEVIATION (C.C./KG.)	STANDARD ERROR (C.C./KG.)
Normal dogs	30	11.56	2.97	0.56
Tracheotomized dogs	35	5.18	1.92	0.33
Tracheotomized rabbits subjected to deep, artificial respiration	32	10.4	4.14	0.76
Tracheotomized rabbits breathing without artificial respiration	43	4.5	3.16	0.55

between the provisional and the calculated dosage-mortality regression lines was tested by calculating chi square (X^2).[7]

The median lethal dose of phosgene for the unanesthetized, tracheotomized dogs was 1.20 mg. per kilogram, with a range of 0.82 to 1.76 mg. per kilogram for P = 0.01. The LD_{50} for the unanesthetized, tracheotomized rabbits was 0.33 mg. per kilogram, with a range of 0.24 to 0.45 mg. per kilogram for P = 0.01, for those which were not subjected to artificial respiration during exposure, and 0.25 mg. per kilogram, with a range of 0.15 to 0.42 mg. per kilogram for P = 0.01, for those subjected to deep artificial respiration. The LD_{50} for the intact rabbits subjected to artificial respiration was 0.20 mg. per kilogram, with a range of 0.16 to 0.24 mg. per kilogram (Table VII).

In Table VIII are listed the *t* values obtained when comparisons are made between the LD_{50}'s of phosgene for the various series of animals reported in this paper. These *t* values and the one obtained by comparing the tracheotomized dogs with the normal dogs previously reported[2] indicate that: (1) eliminating the upper respiratory tract by tracheotomy does not produce a significant change in the LD_{50} for dogs or rabbits; (2) there is a highly significant difference between the LD_{50}'s for tracheotomized dogs and tracheotomized rabbits; and (3) although increasing the depth and decreasing the minute volume of respiration in tracheotomized rabbits tends to decrease the LD_{50}, the decrease is not statistically significant.

Percentage Retention of Inhaled Phosgene.—Calculation of the percentage of inhaled material retained after dosimetric exposure is dependent on the aver-

*Chamber concentration × Correction factor = Mean contact concentration.
†Cc × Correction factor = Corrected Cc.

TABLE VII. LD_{50}'S FOR UNANESTHETIZED, TRACHEOTOMIZED AND INTACT, NORMAL DOGS AND RABBITS EXPOSED TO MEAN PHOSGENE CONCENTRATIONS OF APPROXIMATELY 1.5 MG. PER LITER

EXPERIMENTAL ANIMALS	NUMBER OF ANIMALS	LD_{50} (MG./KG.)	RANGE OF LD_{50} FOR $P = 0.01$ (MG./KG.)	STANDARD ERROR (MG./KG.)
Normal rabbits subjected to deep, artificial respiration	32	0.20	0.16 to 0.24	0.015
Trach. rabbits subjected to deep artificial respiration	32	0.25	0.15 to 0.42	0.052
Trach. rabbits breathing without artificial respiration	43	0.33	0.24 to 0.45	0.042
Normal dogs[2]	30	1.31	1.09 to 1.59	0.097
Tracheotomized dogs	35	1.20	0.82 to 1.76	0.183

TABLE VIII. t^* VALUES OBTAINED WHEN LD_{50}'S OF PHOSGENE FOR TRACHEOTOMIZED, UNANESTHETIZED DOGS AND RABBITS AND FOR NORMAL RABBITS ARE COMPARED BY STUDENT'S t TEST

SPECIES	TRACHEOTOMIZED DOGS	DEEP-BREATHING TRACHEOTOMIZED RABBITS	SHALLOW-BREATHING TRACHEOTOMIZED RABBITS	DEEP-BREATHING NORMAL RABBITS
Tracheotomized dogs	—	*4.97*	*4.65*	*5.29*
Deep-breathing tracheotomized rabbits	*4.97*	—	1.11	0.7
Shallow-breathing tracheotomized rabbits	*4.65*	1.11	—	2.05
Deep-breathing normal rabbits	*5.29*	0.7	2.05	—

* $t = \dfrac{m_1 - m_2}{\sqrt{(E_1)^2 + (E_2)^2}}$ (m = *Median lethal dose.*) (E = *Standard error.*)

age tidal air during exposure.[2] In the intact rabbits with marked inhibition of respiration, many individual breaths could not be identified on the spirometer records, despite artificial respiration. Therefore, the average tidal air could not be estimated accurately, and the percentage retention could not be calculated.

The mean percentage of inhaled phosgene retained by the tracheotomized rabbits subjected to deep artificial respiration was 78.3 per cent, with a standard deviation of 15.8 per cent and a standard error of 2.9 per cent. The mean percentage retention for the shallow-breathing, tracheotomized rabbits was 48.1 per cent, with a standard deviation of 21.0 per cent and a standard error of 3.7 per cent. The t value (6.1) confirms the marked significance of this difference.

The mean percentage retention of phosgene in the tracheotomized dogs was 61.7 per cent, with a standard deviation of 13.8 per cent and a standard error of 2.4 per cent. The t value (4.6) establishes the statistical significance of the difference between this mean and that of 74 per cent previously reported[2] for normal dogs.

DISCUSSION

Boyd and Perry[8] investigated the influence of the nasobuccal pharyngeal filter on the toxicity of phosgene. They reported that, in rabbits and cats but not in dogs, by-passing the upper respiratory tract by tracheotomy before exposure to 0.30 to 0.45 mg. per liter of phosgene for thirty minutes produced markedly greater mortality than was observed in intact animals of the respective species exposed to the same concentration of phosgene. The difference be-

tween the several species was attributed to the fact that little phosgene is removed and thus rendered innocuous by the nasal mucosa of the dog, which is a mouth-breather in warm weather, in contrast to the nose-breathing rabbits and cat.

However, the influence of respiratory volume on the dose of phosgene acquired during conventional chamber exposures cannot be ignored. Inasmuch as there is no decrease in the respiratory intake of tracheotomized animals during exposure to moderate concentrations of phosgene, such animals must inhale more agent during a given period than do intact animals in which contact of phosgene with the nasal mucosa produces marked respiratory inhibition. Since this respiratory inhibition is of variable duration in different species, the species differences which Boyd and Perry have observed in the effect of tracheotomy on mortality following Ct exposure to phosgene for thirty minutes may be due to the fact that in the presence of phosgene, intact rabbits can maintain markedly reduced respiration for long periods, whereas intact dogs do not inhibit respiration significantly for more than a minute or two.[2, 9] Consequently, the course of the influence of the upper respiratory tract on the toxicity of phosgene cannot be evaluated by chamber exposures.

The results of our experiments indicate that the susceptibility of dogs and rabbits to the toxic action of phosgene is not significantly affected by the elimination of the upper respiratory tract by tracheotomy. Since the actual dose of phosgene retained was measured, uncontrolled differences in respiratory intake during exposure could not affect survival. Apparently, the proportion of inhaled phosgene which is absorbed by the upper respiratory tract at the concentrations studied is too small and inconstant to produce statistically significant changes in the median lethal dose, particularly when the variability (statistical range) is great, due either to heterogeneity or to the small size of the sample of the experimental population, or to other factors previously discussed.[2] In this connection it is interesting that, by direct measurement, Cameron, Gaddum, and Short[10] observed that at nominal concentrations of 40, 100, and 500 mg. per m^3 "phosgene passes through the nose without marked loss, although 25 per cent difference in phosgene concentration of air entering and leaving the nose is not uncommon." Later, they[10] found that at higher concentrations, equivalent to those to which our dogs and rabbits were exposed, only 2 to 4 per cent of phosgene was removed by the nasal mucosa of monkeys.

In the tracheotomized dogs the depth of respiration was significantly less and the respiratory minute volume greater than in the normal group. If the toxicity of phosgene increases as respiration becomes deeper and slower, this might have masked a significant change in the LD_{50} after tracheotomy. To explore this possibility further, the LD_{50} was determined in a group of tracheotomized rabbits in which, by means of artificial respiration, the depth of respiration was increased to more than twice that of the first group of tracheotomized rabbits, and the respiratory minute volume was correspondingly decreased. As previously noted, however, although the LD_{50} for the shallow-breathing rabbits was 40 per cent greater than that for the deep-breathing rabbits, the statistical spread for the two groups was too great to permit the demon-

stration of significance in this difference. A larger series with more uniform control of depth and minute volume of respiration might clarify this point further.

A highly significant difference between the percentage of phosgene retained by the deep-breathing and by the shallow-breathing tracheotomized rabbits was demonstrated. An increase in the percentage of inhaled gas retained as the tidal air volume is increased and the rate of airflow is decreased would be expected, since, with increased depth of respiration, a greater proportion of the inhaled vapors comes into direct contact with pulmonary respiratory epithelium and the slower air flows increase time of contact. In the earlier experiments on normal dogs,[2] goats,[3] and monkeys,[4] in which the depth of respiration was not controlled, this relationship was obscured. However, the decreased percentage of phosgene retained by the tracheotomized dogs as compared with the more deeply breathing, normal dogs probably is attributable, in part, to the shallower, more rapid respiration of the former group.

The failure to explain the reported species differences in susceptibility to phosgene by eliminating the upper respiratory tract indicates that as yet undefined factors must be responsible. These data suggest that one such factor may be depth of respiration.

SUMMARY

1. The median lethal dose, the per cent of inhaled agent retained, and the respiratory changes during dosimetric exposure to phosgene were determined, after by-passing the upper respiratory tract by tracheotomy, in thirty-five unanesthetized dogs, in thirty-two unanesthetized rabbits which were subjected to deep, artificial respiration during exposure, and in forty-three unanesthetized rabbits which breathed more shallowly and rapidly without artificial respiration.

2. In addition, thirty-two normal, unanesthetized rabbits were subjected to artificial respiration during dosimetric exposure to a mean phosgene concentration of approximately 1.25 mg. per liter. The median lethal dose for this group was 0.20 mg. per kilogram (standard error = 0.015 mg. per kilogram). Because of leakage of the facepiece, the dosimetric technique described is not recommended for use in untracheotomized rabbits.

3. The median lethal dose for the dogs which were exposed to an average phosgene concentration of 1.48 mg. per liter was 1.20 mg. per kilogram (standard error = 0.18 mg. per kilogram). This is not significantly different from the LD_{50} (1.31 mg. per kilogram) previously reported for untracheotomized dogs exposed at approximately the same concentration.

4. The median lethal dose for unanesthetized, tracheotomized rabbits was 0.25 mg. per kilogram (standard error = 0.052 mg. per kilogram) for a group of thirty-two, which was subjected to deep, artificial respiration during exposure to a mean phosgene concentration of 1.52 mg. per liter, and 0.33 mg. per kilogram (standard error = 0.042 mg. per kilogram) for the group of forty-three, which breathed more shallowly and rapidly without artificial respiration during exposure to a mean phosgene concentration of 1.30 mg. per liter. The decrease in the LD_{50} produced by the increase in depth and decrease in velocity of respira-

tory flow is, however, not statistically significant. Moreover, as in dogs, the LD_{50}'s in tracheotomized and in intact rabbits are not significantly different. Therefore, since the susceptibility of both dogs and rabbits to phosgene apparently is not significantly affected by by-passing the upper respiratory tract during exposure, previously reported differences in the resistance of these two species to phosgene poisoning are not explained by this procedure.

5. The mean percentages of inhaled phosgene retained were as follows: 61.7 per cent (standard deviation = ± 13.8 per cent; standard error = ± 2.4 per cent) for the tracheotomized dogs; 78.3 per cent (standard deviation = ± 15.8 per cent; standard error = ± 2.9 per cent) for the deep-breathing, tracheotomized rabbits; and 48.1 per cent (standard deviation = ± 21.0 per cent; standard error = ± 3.7 per cent) for the shallow-breathing, tracheotomized rabbits. No data on percentage retention could be obtained in the intact rabbits. The mean percentage retentions for the tracheotomized dogs and the shallow-breathing, tracheotomized rabbits are significantly less than that of 74 per cent previously observed for a group of untracheotomized dogs and that for the deep-breathing rabbits, respectively. Since in the tracheotomized dogs and in the shallow-breathing, tracheotomized rabbits the mean tidal air was much smaller and the mean minute volume was greater than in the normal dogs and in the deep-breathing rabbits, respectively, these differences demonstrate the influence of depth and minute volume of respiration on the percentage of the agent retained after inhalation.

6. In contrast to intact dogs, goats, monkeys, and rabbits, none of the tracheotomized rabbits or dogs, in which contact with inhaled vapors was limited to the respiratory tract below the larynx, exhibited any signs of respiratory inhibition or irritation during exposure to phosgene.

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CHROMOGENIC REACTIONS TO THE HALOGENATION OF PREGNANCY URINE

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GERMAN patent No. 680223 advances the claim for a pregnancy test requiring only a few minutes to perform and displaying a high degree of accuracy. The reaction is dependent on the production of a rose color when a solution of iodine is added to pregnancy urine, the color being characterized by its solubility in isoamyl alcohol. Schales and Schales[1] have made an effort to identify the substance responsible for this phenomenon, but we know of no other reports on the procedure.

TECHNIQUE

The iodine reagent was prepared by adding 1.5 Gm. of iodine and 2.5 Gm. potassium iodide to 100 c.c. of water.[1] Using glacial acetic acid and/or NaOH, the urine is adjusted to a pH of 6.0 to nitrazine paper. Five cubic centimeters are placed in each of two test tubes and the tubes immersed for thirty seconds in a beaker of boiling water. As soon as the tubes are removed, 0.2 c.c. of the iodine reagent is added to one and 0.4 c.c. to the other specimen. The solutions are agitated gently for five seconds, and 2 c.c. of isoamyl alcohol is added to each. After shaking vigorously for a moment the tubes are allowed to stand until the alcohol has stratified above the urine. A rose or pink color in the alcohol layer of either tube is read as positive. All other color changes or no color are read as negative.

Results.—The test was carried out 300 times. In eight cases (2.7 per cent) the reading was doubtful. Of the remaining 292 patients, 203 were pregnant, and the result was positive in 159 (78.3 per cent). In the eighty-nine nonpregnant patients, negative reports were obtained in sixty-nine (77.5 per cent). This same material is rearranged in Table I to provide more information from the clinician's viewpoint. From this it can be seen that given a positive report there is about an 11 per cent chance of error, while the negative report carries almost a 40 per cent error.

Comment.—The claims made for the test cannot, on the basis of this work, be substantiated, and this degree of inaccuracy rules out the procedure as an aid in the diagnosis of pregnancy. The routine checking of such a claim, however, while a necessary task, does not completely eliminate the reaction from further consideration. In the first place, it represents a chromogenic phenomenon which occurs more frequently in the pregnant than in the nonpregnant woman, and from this point of view it merits consideration. Furthermore, a certain amount of interest is attached to the test since it is the third time that halogenation of the urine has been advanced as a color reaction for pregnancy.

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TABLE I

Positive Tests (179)	
Patient pregnant	88.8%
Patient not pregnant	11.2%
Negative Tests (113)	
Patient pregnant	39.0%
Patient not pregnant	61.0%

Friedrich[2] advocated a test based on the addition of HCl to urine and the development of a dark red-brown color, while the histidine test, which has had a more extensive trial,[3] rests on the color developed on the bromization of pregnancy urine. In neither the iodine nor the hydrochloric acid reactions has the responsible chromogenic substance been determined, and the question arises as to the identity or parallelism between these procedures.

The Color Change.—There is a diminution in the amount of color developed as the pH of the urine tested shifts either way from the optimum point of six. As noted by Schales and Schales,[1] charcoal will adsorb the chromogen, but other adsorption and ion-exchange media (aluminum earth, zeolites, and synthetic resins) did not remove it. Preliminary treatment of the urine with permutit, in fact, intensifies the final color somewhat.

The chromogen itself is not heat labile, but leaving the tubes immersed in the boiling water bath for two minutes or longer elevates the temperature to a point where the color will not develop. On the other hand, the reaction will not occur when the iodine reagent is added to urine at room temperature. The solubility in isoamyl alcohol refers only to the final pigment and does not apply to the substance responsible for the reaction. Thus, preliminary extraction of the urine with this alcohol, either at room temperature or after gentle heating, does not alter the color developed.

The relationship between the results of the test and the duration and nature of the pregnancy is not consistent. The distribution of false negatives in the various trimesters is equal, and the presence or absence of albuminuria or lactosuria does not influence the results. Consecutive daily tests during the immediate puerperium were run on ten patients who had had positive reactions at the time of delivery. Six of these turned negative between the fourth and the sixth post-partum day, while the remainder continued to excrete the chromogen until the time of discharge. One patient, however, after two negative reports on the fourth and the fifth post-partum days again excreted the chromogen on the sixth day, and in the course of selecting these cases, a patient was discovered who had a negative test at delivery but turned positive on the first post-partum day. These results stress the inconsistency of the test with relation to the status of the pregnancy.

RELATIONSHIP TO OTHER HALOGENATION PROCEDURES

Hydrochloric Acid Test.—A few drops of 25 per cent HCl are added to 1 c.c. of urine having a specific gravity of 1.015. After heating a few minutes a dark-brown or a deep red-brown color is considered positive, while a light brown or other color is reported negative. With this reaction Friedrich reported 100 per cent accuracy.[2]

In a series of sixty tests performed by us, thirty on pregnant and thirty on nonpregnant urines, the Friedrich test produced 20 per cent false positive and 50 per cent false negative results. Coincidental performance of the iodine test on these same urine specimens revealed no identity or parallelism in the reaction of the two chromogenic substances. The combined results obtained from these simultaneous procedures (that is, both tests positive, both negative, or a conflicting result) could not be correlated with the clinical diagnoses and did not improve the accuracy of the laboratory reports.

While their behavior with various adsorption media differs, the most striking dissimilarity between the two chromogens lies in their reaction to heat. The rose color cannot be developed when the temperature of the urine is above 90° C., whereas the color developed on adding HCl is accentuated by prolongation of the heating.

Histidine.—That most nontoxemic pregnant women excrete histidine has long been known. That the phenomenon is not consistent enough to provide a pregnancy test is also acknowledged. The recent excellent work of Page[4] has shown that the reaction is apparently due to a lowering of the renal threshold for histidine during pregnancy.

Histidine gives a violet color on bromization, which has formed the basis for most of the histidine tests described. A solution of histidine hydrochloride when mixed with the iodine reagent does not, however, yield the characteristic rose color, and the behavior of the rose pigment toward various adsorption media differs from that of histidine. The rose-color reaction under discussion apparently is not dependent on nor related to the presence or absence of histidine.

In the course of comparing the two procedures, however, it was noted that whenever both tests were positive on the same sample of urine the patient was invariably pregnant. The coincidence of a positive rose-color reaction and a positive histidine reaction carried an almost negligible diagnostic error. The question was raised, accordingly, as to the frequency with which, in a series of cases, the two tests coincided with positive reactions.

The bromization technique of Page[3] is too sensitive for qualitative purposes, and with it almost all urines are positive. The procedure was therefore modified to "desensitize" it by treating the urine initially with Lloyd's reagent and omitting the addition of sodium urate. One gram of Lloyd's reagent was used for each 10 c.c. of urine and filtered out after five minutes. Three-tenths of a gram of charcoal was used in the step removing urinary pigments, and, since both adsorption media remove some histidine, this technique yielded positive results only in the face of a high concentration of histidine.

Results.—The rose-color reaction and the histidine test were carried out on 253 samples of urine. Both tests were positive in ninety-one cases (36 per cent) and negative in 73 (29 per cent), while the results of the two procedures disagreed in the remaining eighty-nine cases (35 per cent). Of the ninety-one patients with whom both procedures were positive, ninety (98.8 per cent) were pregnant and one was nonpregnant. However, when both tests were negative there was a 22 per cent error attendant on the diagnosis of nonpregnant, and in the face of conflicting reports from the two tests no diagnosis could be made

at all. Of the 253 patients, 176 were pregnant, and the "double positive" occurred in only ninety (51 per cent).

Comment.—In so far as the hydrochloric acid reaction goes, it is doubtful that there is a single chromogenic substance responsible for the color change. Actually the procedure is apparently a modified acid hydrolysis, and while it is true that the hormone content of a urine specimen can effect the color developed on acid hydrolysis, it is also obvious that the initial pigment content of the urine as well as its nonhormonal salts will have a decided influence.

In contrast to this, the development of the rose color seems to rest on a definite chromogenic substance. While the exact nature of the substance is undetermined, it would appear to be neither hormonal nor specifically related to pregnancy. The degree of diagnostic accuracy for the simultaneous occurrence of positive readings in the iodine and bromine reactions is remarkable, although the fundamental basis for such a finding remains obscure. Aside from presenting an avenue for investigation, however, the combined procedures have a dubious value, and it is questionable that there is any immediate clinical application for the double test. Although the two determinations can be carried out in twenty minutes, two out of every three performed yielded a report from which no conclusion could be drawn. In one case out of every three (in this series) a result was obtained which provided over 98 per cent accuracy in the diagnosis of pregnancy.

CONCLUSION

1. A rose color, soluble in isoamyl alcohol, is produced when an iodine solution is added to some specimens of urine. While this reaction occurs more frequently in the pregnant than the nonpregnant woman, the claim that it provides a suitable diagnostic test for pregnancy cannot be substantiated on the basis of the studies herein reported.

2. The color reaction which does take place is not related to the HCl reaction proposed by Friedrich as a pregnancy test nor to the histidine content as determined by bromization.

3. The observation was made in the course of this study that a coincidental positive rose-color and positive histidine test was associated with pregnancy in 98.8 per cent of the cases. In contrast to this high degree of specificity, however, the sensitivity is only 50 per cent, and the excretion of the rose chromogen bears no consistent relationship to either the duration or the nature of the pregnancy.

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SOME OBSERVATIONS ON SEVERE DIABETIC KETOSIS TREATED WITH GLUCOSE AND INSULIN

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THE present report is concerned with some observations on acetone bodies and glucose in blood and urine, which were made on a group of patients with diabetic coma, or threatened coma, who were treated with glucose and insulin. The results seem to bear on some of the unsettled problems in this field.

METHODS

Acetone and total acetone bodies were determined by the method of Greenberg and Lester.[1] The quantities of reagents and sample solutions were increased fivefold, so that a volume of 10 c.c. of carbon tetrachloride extract was obtained for reading in the Evelyn colorimeter. The digestion for total acetone bodies was carried out under a reflux condenser with standard taper ground-glass connections.

Qualitative clinical tests of the acetone bodies in the urine were made by: (1) the nitroprusside technique of Rabinowitch[2]; and (2) a modified Gerhardt technique: boil about 10 c.c. of urine for two minutes; if a cloud develops, add a drop of strong HCl, and if it is still turbid, filter. Transfer 4 c.c. of the boiled urine to one tube and 4 c.c. of the fresh urine to a similar tube. To each add 2 c.c. of a solution containing 50 Gm. $FeCl_3$ and 12 c.c. concentrated HCl per liter. As ordinarily carried out, the ferric chloride test is difficult to assay in the presence of salicylate, especially if a little protein is also present. with the technique described here, relatively small concentrations of diacetic acid may be detected by a difference of color intensity in the two tubes.

Quantitative determinations of glucose were made, at first by an adaptation of the technique of Hoffman,[3] subsequently by the Nelson-Somogyi[4] colorimetric procedure.

The Ketosis in Relation to the Diagnosis of Diabetic Coma.—Two examples of diabetic coma are provided by Cases 3 and 4 in Table I, with the urine giving a negative reaction with ferric chloride and only a mild reaction with nitroprusside. The concentrations of acetone in blood and urine are similar in these cases, as in all of the others; however, the total acetone bodies of the urine in these two cases are at levels lower than those found in the blood. These observations are, therefore, in confirmation of others[5, 6] which indicate that too much reliance should not be placed on tests for diacetic acid in the urine in the diagnosis of diabetic coma.

On the admission specimens the free acetone content of the blood ranges from 20 to 32 per cent of the total acetone. These figures are of the same order as those reported by Marriott.[7] Since the free acetone of the blood always amounts to a substantial proportion of the total acetone and since the acetone of the alveolar air bears a mathematical relation to the blood acetone, a test for acetone in alveolar air should give an index of the extent of the ketosis. A simple clinical method for the estimation of acetone in alveolar air has been described previously.[8]

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TABLE I. SHOWING CHANGES IN BLOOD AND URINE OF PATIENTS WITH SEVERE DIABETIC ACIDOSIS DURING TREATMENT WITH GLUCOSE AND INSULIN

		BLOOD			URINE				
		QUANTITATIVE MG. %			QUANTITATIVE MG. %		QUALITATIVE		
	HOURS AFTER BLOOD NO. 1	ACETONE	TOTAL ACETONE BODIES	GLUCOSE	ACETONE	TOTAL ACETONE BODIES	$FeCl_3$	NITROPRUSSIDE	SUGAR
Case 1									
Blood and urine No. 1	0	23.7	90.6	889	22.2	319.2	+++	++++	++++
50 units insulin S.C.	0								
1 liter 5% glucose-saline I.V.	2								
Blood and urine No. 2	4	23.3	68.3	668			++	++++	++++
50 units insulin S.C.	4								
1 liter 5% glucose-saline I.V.	6								
Blood and urine No. 3	12.5	9.2	32.4	363	10.8	57.8	+	+++	++++
Case 2									
Blood and urine No. 1	0	5.8	28.1	358	5.1	63.3	+	+++	++++
50 units insulin S.C.	0								
1 liter 5% glucose-saline I.V.									
Blood and urine No. 2	10.5	2.5	14.4	122	3.1	15.4	Neg.	+	+++
Case 3									
Blood No. 1	0	25.6	114	680	26.2	63	Neg.	++	++++
50 units insulin S.C. plus 50 units I.V.	0								
1 liter 5% glucose-saline I.V.	2								
Blood No. 2 and urine No. 2	3	24.4	67	615	22.4	71	Neg.	++	++++
Case 4									
Blood No. 1	0	29.4	91	478	29.7	86	Neg.	++	++++
150 units insulin S.C.	0								
3 liters 5% glucose-saline I.V.	6								
Blood No. 2 and urine No. 2	12	6.4	13	230	6.9	14	Neg.	Trace	++
Case 5									
Blood and urine No. 1	0	33.2	111.0	641	37.6	741.0	++++	++++	++++
50 units insulin I.V plus 150 units S.C.	0								
2 liters 5% glucose-saline I.V.	3.5								
Blood and urine No. 2	4.5	31.9	59.0	361	37.4	168.0	++	++++	++++
50 units insulin S.C.									
50 Gm. glucose plus orange juice orally	6								
Blood and urine No. 3	10.5	24.1	36.4	75	24.2	44.8	Neg.	++	+

(Table I cont'd on following page)

TABLE I.—CONT'D

	HOURS AFTER BLOOD NO. 1	BLOOD QUANTITATIVE MG. % ACETONE	BLOOD QUANTITATIVE MG. % TOTAL ACETONE BODIES	BLOOD QUANTITATIVE MG. % GLUCOSE	URINE QUANTITATIVE MG. % ACETONE	URINE QUANTITATIVE MG. % TOTAL ACETONE BODIES	URINE QUALITATIVE $FeCl_3$	URINE QUALITATIVE NITROPRUSSIDE	URINE QUALITATIVE SUGAR
Case 6									
Blood and urine No. 1	0	32.2	102.0	930			++++	++++	++++
150 units insulin I.V. plus 200 units S.C.	0								
500 c.c. plasma plus 500 c.c. saline I.V.									
3 liters 5% glucose-saline I.V.	10								
Blood and urine No. 2	16	13.8	39.5	428			Neg.	++	++++
Case 7									
Blood and urine No. 1	0	21.5	94.0	507	27.8	436.5	+++	++++	++++
100 units insulin S.C.	0								
2 liters 5% glucose-saline I.V.	6								
Blood and urine No. 2	14	17.8	54.7	64	19.0	77.0	+	+++	+

Saline was administered subcutaneously to each of these patients during the first few hours of treatment. The insulin employed was "regular" soluble insulin. The figures for acetone bodies represent acetone equivalents.

On the Use of Glucose in the Treatment of Coma.—This has been a very controversial subject. That administration of glucose is not necessary in the treatment of coma, providing the laboratory facilities are such that blood chemistry can be obtained as desired at any time of the day or night, is certain from the reports of Joslin[9] and Root.[10] Among the objections to the use of glucose which have been suggested are the following: (1) Glucose neutralizes insulin. It is true that glucose will prevent insulin hypoglycemia, but according to Drury and Palmer,[11] the activity of insulin is greater at high blood sugar levels. (2) Intravenous administration of glucose solutions will aggravate diuresis and dehydration. This is true for strong glucose solutions in distilled water but not for 5 per cent glucose in saline. (3) Glucose administration interferes with the use of blood sugar as a basis of control. This is a valid objection. However, the objection would be a more important one if the level of blood sugar could be taken as an accurate index of the extent to which the metabolic disturbance is corrected. That this is not always true is shown by the results in Cases 2, 5, and 7. In each of these, the level of the blood sugar had returned to the normal range when the last blood was taken, but the ketosis in each instance was far from complete correction. Accordingly, tests which indicate the extent of the ketosis are suggested as an alternative basis of control. Determination of the plasma bicarbonate may be substituted for the determination of total acetone bodies. Although lowering of bicarbonate is not due entirely to ketone acids, as pointed out by Martin and Wick,[12] the lowering of the bicarbonate level is considered by Joslin and co-workers[5] as a reliable index of the diabetic acidosis, and improvement in the plasma bicarbonate has been suggested as a basis of control by Almy, Swift, and Tolstoi.[13] Except for cases with suppressed ex-

cretion, the intensity of the ketosis is indicated in a satisfactory manner by the tests for diacetic acid in the urine. As illustrated by the results in the table, the test with ferric chloride becomes negative, first, and then fading of the test with nitroprusside follows.

The following are among the advantages associated with the use of glucose: (1) The administration of glucose lessens the chance of hypoglycemia. This is particularly important in the elderly patient with diabetes with coronary disease. It is well known[14] that such patients are prone to attacks of angina with hypoglycemia or even with a sudden drop of blood sugar from a chronic high level to the normal range. In Cases 2, 5, and 7, the return to normal would have been considered too rapid for elderly subjects; more glucose would have been administered. (2) The patient in diabetic coma is in need of carbohydrate.[15] His stores of glycogen are to a certain extent depleted, and intravenous administration of glucose is the most feasible method for carbohydrate replacement and for providing nourishment until he is able to take food by mouth. According to the calculations of Joslin,[9] who minimizes the need, the total carbohydrate of the patient in coma may be depleted to an average extent of about 30 Gm., and Joslin concedes the need for carbohydrate to cover half the caloric requirement for the first day, which would amount to about 175 Gm. for a man weighing 70 kilograms. In other words, such a patient should have at least 200 Gm. of glucose in the first twenty-four hours. The patients who were the subjects of this study received from 100 to 200 Gm. within the first few hours. Certainly this was not too much. In each instance, by the time the last blood sample was taken, the administered glucose had been disposed of and part of the patient's blood sugar as well. In Cases 5 and 7, a state of mild hypoglycemia had developed.

In view of the observed results, it is suggested: that the patient in diabetic coma should receive glucose in quantities equivalent to at least half the caloric requirements; that administration of insulin might be controlled by tests which indicate the intensity of the ketosis; and that determinations of sugar in blood and urine be carried out in order to safeguard against hypoglycemia.

SUMMARY

Observations have been made on changes in the concentrations of sugar and acetone bodies in blood and urine during the treatment of diabetic coma with insulin and glucose. From the observed results, suggestions have been made concerning the appropriate quantities of glucose which should be administered and concerning appropriate tests for diagnosis and control.

Acknowledgment is made for clinical assistance to Dr. J. M. Martin, Dr. J. L. Mulherin, Dr. Phyllis Johnson, Dr. J. L. Lokey, and Dr. J. W. Hurst.

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THE EFFECT OF GLYCEROPHOSPHATE ON BLOOD COAGULATION AND ON BLOOD CLOT RETRACTION

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THE anticoagulant effect of soluble glycerophosphates has been previously mentioned.[1] Since the solubility of calcium glycerophosphate, like the citrate, has a negative temperature coefficient, it seemed likely that calcium glycerophosphate would be poorly ionized and that glycerophosphate might inhibit coagulation by calcium ion depressant effect. This proved to be the case. Glycerophosphated plasma is readily coagulated by the addition of calcium chloride, sulfate, or dibasic phosphate, and whole glycerophosphated blood showed an unusual delay in spontaneous hemolysis on storage. Glycerophosphate found a limited application as an anticoagulant for blood specimens that had to be transported for long distances under unfavorable conditions prior to special serologic examination; however, since a final concentration of 2 per cent glycerophosphate in the plasma was found necessary for complete coagulation inhibition, the utility of this anticoagulant was limited by expense.

When less than 2 per cent glycerophosphate was added to freshly drawn blood, coagulation was delayed; however, after the advent of the latter, clot retraction appeared to be accelerated. Because there are clinical conditions in which clot retraction is deficient, this effect was further studied.

EXPERIMENTAL

Eastman's sodium glycerophosphate (52 per cent alpha) and Merck's beta preparation were used. Both gave essentially the same results. After dissolving the substance in water, the solutions were brought to pH 7.4 by the addition, dropwise, of concentrated HCl.

Blood was collected directly from the arm vein of human subjects by the insertion of a 3 inch, 20 gauge needle and allowed to flow directly down the glycerophosphated, solution-moistened wall of a graduated centrifuge tube containing measured quantities of 5, 10, or 20 per cent sodium glycerophosphate solution. During collection the centrifuge tube was continuously agitated by tapping the bottom to facilitate admixture with the anticoagulant solution, which, because of its high viscosity and specific gravity, otherwise tends to stratify at the bottom.

Coagulation time was measured by the Lee-White method[2] and clot retraction time by that of Macfarlane,[3] with the necessary modification that these determinations were made within the centrifuge tube into which the blood collection had been made.

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RESULTS

While a final concentration of 2 per cent or over of sodium glycerophosphate was found necessary to prevent coagulation indefinitely in most samples tested, in some, as little as 0.04 per cent would cause sensible inhibition. In other samples, 1 per cent glycerophosphate would delay coagulation for several hours. More constant, however, is the effect of subminimal anticoagulant quantities of sodium glycerophosphate on clot retraction time which is definitely diminished, as shown by representative experiments in Table I. Not amenable to quantitative representation is the fact that clots in glycerophosphated blood appear to shrink more rapidly and to attain a smaller final volume than in the control blood samples.

TABLE I. EFFECT OF SODIUM GLYCEROPHOSPHATE ON COAGULATION AND CLOT RETRACTION TIME

SAMPLE	GLYCEROPHOSPHATE (PER CENT)	CLOTTING TIME (MINUTES)	CLOT RETRACTION TIME (MINUTES)
Be	0.00	3.5	70
	0.25	5.0	40
	0.50	6.0	25
Myk	0.00	3.5	55
	0.10	4.5	35
	0.20	6.0	30
	0.50	9.0	20
Gi	0.00	5.0	80
	0.50	7.0	45
	1.00	8.5	30
	1.50	14.0	40
	2.00	No clot	

It might be mentioned that in the blood of a patient with terminal acute myeloid leucosis, with extensive purpura and a thrombocyte count of 28,000, the addition of 0.25 per cent sodium glycerophosphate did not induce clot retraction which was found to be absent both in the glycerophosphated and homologous control blood sample at the end of forty-eight hours. Also worthy of mention is the confirmation of the fact, previously noted, that blood samples containing completely anticoagulant concentrations of glycerophosphate, after a month of icebox storage, show barely a tinge of hemolysis.

SUMMARY

The addition of sodium glycerophosphate to shed blood in concentrations of 2 per cent or over completely prevents coagulation. Inhibition of coagulation is usually evinced by concentrations of 0.10 per cent and may be evinced in some samples by as little as 0.04 per cent. Concentrations which permit clotting cause an acceleration of clot retraction.

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BLUE FLUORESCENT SUBSTANCE IN URINE

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SOME of the carcinogenic hydrocarbons have very distinctive fluorescent spectra. These spectra consist of sharp bands in the blue region. This property has aided in the detection and isolation of these substances. The separation of benzpyrene from coal tar by Cook and co-workers[1] is a good example of this kind of investigation.

In the present paper is reported the detection of a substance in urine which has two bands in its fluorescent spectrum, with wave lengths of approximately 4100 A and 4400 A. Furthermore, this fluorescent property has allowed the substance to be traced through several chemical reactions. The physical and chemical properties thus far determined indicate that this substance is closely related to known carcinogenic agents.

The concentration of the substance was performed in such a manner that chemical changes in the urine would be avoided. The urine was placed in a low-temperature evaporator and dried from the frozen state. The construction of this evaporator already has been described.[2]

The residue was washed twice with benzene. The benzene washings from 100 liters of urine were collected and the benzene removed in the evaporator. This yielded a dark fatty residue, which was chilled in an ice bath and washed with ethyl alcohol. At this temperature the alcohol dissolved little of the fat but removed the fluorescent material, giving a dark solution.

The alcoholic solution was then reacted with paracarboxyphenylhydrazine. The latter is a ketone reagent that does not combine with the fluorescent substance but does combine with other substances that are present. One gram of paracarboxyphenylhydrazine was added to 100 c.c. of the alcoholic solution, and the flask, fitted with a reflux condenser, was placed in boiling water for one hour. The contents of the flask were then evaporated to dryness in the evaporator.

Two hundred cubic centimeters of 4 per cent potassium carbonate were added to the residue, and the mixture was heated gently for thirty minutes. This formed the potassium salt of the excess paracarboxyphenylhydrazine and all compounds formed from its reaction.

The water mixture was reduced to dryness in the evaporator and the residue washed with benzene. This benzene solution was then evaporated to dryness producing a few milligrams of oily material. This is the purest state in which the fluorescent substance has been produced.

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The fluorescent substance has been observed in all samples of urine that have been examined. Calf urine is the richest source thus far found. In the case of calf urine, the bands are clearly visible in the fluorescent spectrum of the first benzene washings, showing that this substance is native to the urine. With human urine, it is probably necessary to carry through the chemical reactions outlined before the bands become visible.

Molecules of this type may have a bond structure that will form an addition compound with maleic anhydride. This Diehls-Alder reaction is exhibited by methylcholanthrene.[3] The addition of maleic anhydride to a fluorescing solution of methylcholanthrene quenches the fluorescence instantly. The same effect is obtained with the unknown substance. It will be noted that the unknown substance is soluble in benzene and does not react with alkalies, paracarboxyphenylhydrazine, or digitonin.

Fig. 1.—Fluorescent spectrograms. *1*, Methylcholanthrene; *2*, unknown substance.

The fluorescent properties of a considerable number of biologic substances have been examined. None of them correspond to the unknown substance. From our experience, a natural product giving fluorescent bands in the blue would be a very rare material.

It is thought that this is a new substance whose nature should be determined regardless of its relation to cancer. A few mice were injected with the substance without causing tumors. However, this work may not be conclusive.

SPECTROSCOPIC EXAMINATION

The fluorescent light from the solutions is weak, so that it is wise to provide a strong source of ultraviolet light and photograph the spectrum with a spectrograph of large aperture. Apparatus that had been used previously for the fluorescence of porphyrins[4] was readily adapted to the present work.

The light source was an arc of National "C" carbons operated at 40 amperes. A Corning filter No. 586 transmitted a narrow band of ultraviolet light around 3800 A. A water cell placed between the arc and the filter allowed the arc to be operated within 8 cm. of the solution. The fluorescing solution was held in a test tube placed directly against the slit of a Steinheil spectrograph. Exposure times on the plates were about five minutes each.

Commercial solvents are likely to contain sufficient fluorescent material to obscure the results. Satisfactory benzene was obtained by washing the commercial product with concentrated sulfuric acid and then distilling twice the

benzene from the frozen state in the evaporator. The ethyl alcohol was a product purified in the Department of Biochemistry.

The present results were obtained over a period of time before their interruption by the war. The work was carried as far as the available resources would allow. The Department of Physics kindly made the Steinheil spectrograph available. It is also a pleasure to acknowledge valuable suggestions contributed by Dr. Rosalind Klass and Mrs. H. C. Brown, who have worked on the problem.

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LABORATORY METHODS

A SIMPLE METHOD FOR ASSURING ACCURACY IN DETERMINATION OF OXYGEN CONSUMPTION BY THE BENEDICT-ROTH APPARATUS

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IN CASES where breathing is irregular it is difficult to trace the "oxygen line" accurately. In some graphs two different lines, representing widely varying oxygen consumptions, can be traced, either of which can be considered the correct one. To obviate this difficulty, a simple method has been devised; it is based on the following considerations: The lung and spirometer form a closed system from which oxygen is removed by absorption through the lung. This system has two compartments, the respiratory tract and the spirometer. At each respiratory movement the relative proportion of gas in each compartment varies; therefore, in order to appreciate the total decrease in volume, it is necessary to measure the gas contained in the spirometer at the same moment of the respiratory cycle. Usually this is done by tracing a line through the points corresponding to normal expiration, when the lung contains only reserve (supplemental) and residual air. The volume of reserve air varies with the depth of expiration, a fact which introduces a variable in the proportion of gas contained in the spirometer, having no relation to the total volume. The residual air, on the other hand, remains constant in the conditions of the experiment; therefore, if only this part of the total volume of gas remains in the respiratory tract, variations in the spirometer volume will correspond accurately to changes in total volume; that is, to oxygen consumption.

PROCEDURE

While the patient is breathing normally into the spirometer, he is instructed to make a deep expiration. It is important to assure the maximum possible emptying of the lung, so that all the reserve (supplemental) air will be expelled into the spirometer; for this the subject must be stimulated to go on expelling air until the expiratory line flattens out. This is repeated once or twice at two- to six-minute intervals. The "oxygen line" is traced by joining the points of extreme expiration. In Figs. 1 and 2 the "oxygen line" obtained by tracing it at the end of normal expiration was parallel to that obtained by joining the points of extreme expiration. Also shown in Fig. 2 is the constancy of residual air, since the three points of extreme expiration are in a straight line. In both cases, as in many others with regular respiratory rhythm, there was no difference in the oxygen consumption values determined by both methods.

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Out of forty-two cases of normal, hyperthyroid, and hypothyroid subjects taken at random, the difference between the oxygen values given by both methods was less than ±0.5 cal. per m^2h in twenty-seven; it was between 0.5 and 1 cal. in six cases, 1 and 2 cal. in five cases, 2 and 3 cal. in three cases, and in one case the method proposed gave a figure 4 cal. below that obtained by the usual method (Fig. 3).

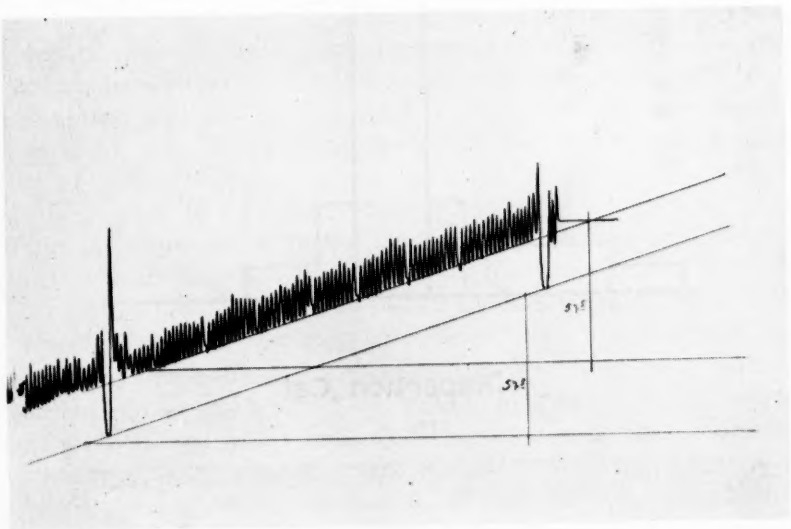

Fig. 1.

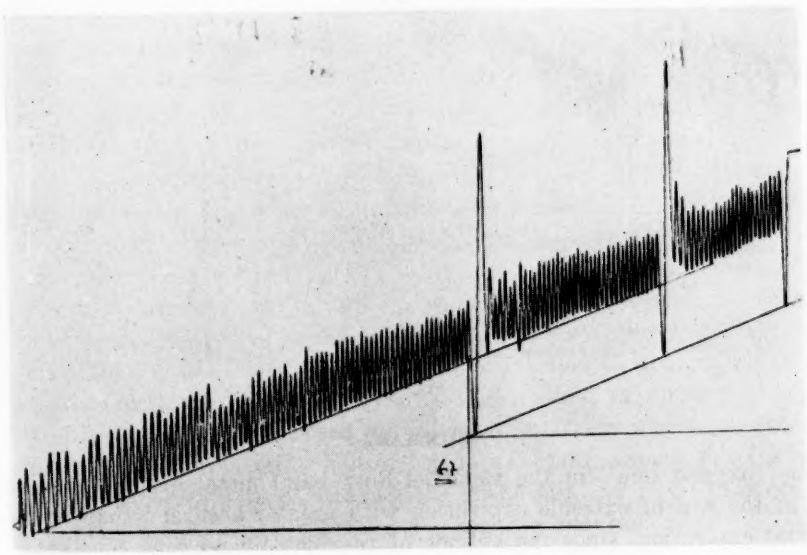

Fig. 2.

In Fig. 4 is shown a respiratory curve that cannot be used to determine oxygen consumption by the normal method because of irregular respiration. The oxygen consumption as determined by the method proposed was 34.8 cal.

m^2h and 35.5 cal. m^2h by the Tissot-Haldane open circuit method. A similar agreement between the results obtained by both these methods was registered in five cases.

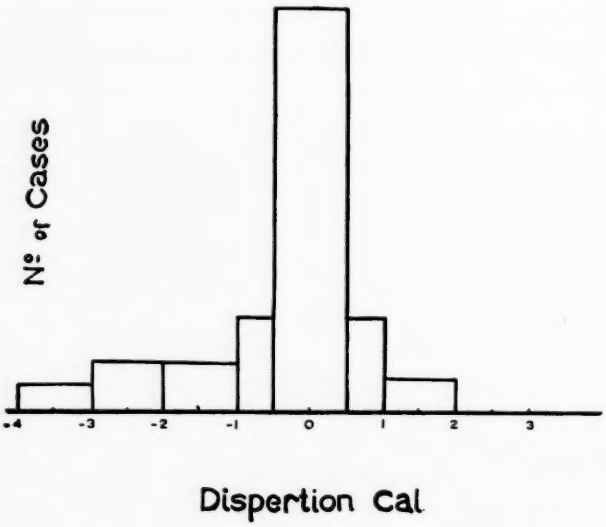

Fig. 3.

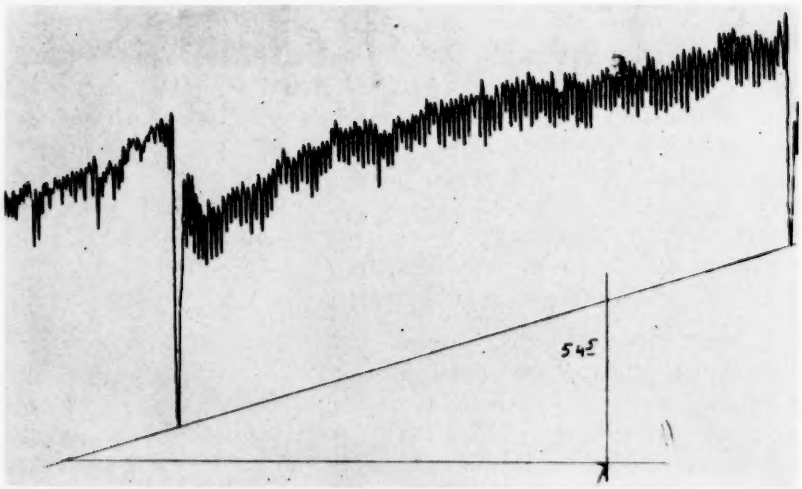

Fig. 4.

SUMMARY

The "oxygen line" in the Benedict-Roth basal metabolism method can be traced at the end of extreme expiration with greater accuracy than at the end of normal expiration, since the volume of residual air remains constant; while the volume of residual plus reserve (supplemental) air varies with the depth of respiration. Accurate oxygen consumption determinations can be made with this method in cases with irregular respiratory rhythm in which by the usual method it is impossible to do; this often occurs in cases of hyperthyroidism in which basal metabolic rate determinations are of primary importance.

A SIMPLIFIED METHOD FOR THE ESTIMATION OF SODIUM

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THE method of estimating the sodium content of urine or blood as described in this paper was developed in the course of a study of the effectiveness of various diuretics. In many of the published articles chloride excretion has been used as an index of sodium loss, but there have been relatively few studies on the ability of various diuretics to cause excretion of sodium. An investigation of the diuretic action of ammonium chloride made it necessary to use a method of determining sodium rather than chloride. A preliminary trial of several of the sodium methods indicated the need of simplification before they could be adapted to routine use. To meet this need a simplified technique was developed and is presented herein as a procedure worthy of use as a routine test.

Sodium was first determined quantitatively by precipitation with uranyl zinc acetate by Barber and Kolthoff.[1, 2] Butler and Tuthill[3] applied this method to biologic solutions and found that interfering substances, such as proteins and phosphates, could be eliminated before the sodium was precipitated. Salit[4] modified the procedure by the addition of alcohol to obtain a quantitative precipitation of the triple salt. The triple salt was then caused to react with potassium ferrocyanide to develop a reddish brown color, the intensity of which was determined in a colorimeter. In our experience, the pH of the solution and the time of color development had to be closely controlled. Weinbach[5] adopted the alcoholic precipitation method of Salit but preferred to titrate the triple salt against 0.02 N sodium hydroxide. Since amphoteric hydroxides were formed in this titration, the end point was not sharp.

Arnold and Pray[6] described a colorimetric method for the determination of sodium. However, the color change in their method required the use of a spectrophotometer. Caley and Foulk[7] used uranyl magnesium acetate to precipitate sodium as a triple salt. They found that when the triple salt was dissolved, the color intensity of the solution was proportional to the concentration. It occurred to us that since the uranyl ion made the reagent a brilliant yellow, the supernatant solution should lose color in proportion to the amount of triple salt that was precipitated. This possibility was realized in subsequent tests, so the method described here is based on the color intensity of the reagent after precipitation of the triple salt. Use of the supernatant reagent has the advantage of eliminating the steps of washing the triple salt and any subsequent procedures of weighing, transfer, color development, or titration.

Reagents.—The uranyl zinc acetate was prepared in the proportions given by Weinbach. However, it was found that the uranyl acetate (77 Gm.) dis-

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solved more quickly in 800 c.c. of water plus 14 c.c. glacial acetic acid. The zinc acetate (231 Gm.) was then added in small portions which dissolved very quickly. The final 7 c.c. of acetic acid was added and the solution made up nearly to volume. After cooling and standing overnight it was made up to final volume (1 liter). This reagent was equally as satisfactory as that prepared by dissolving the uranyl and zinc acetates separately and combining the hot saturated solutions.

Standard solutions of chemically pure sodium chloride were made up over a range of 1.0 to 10.0 mg. per cubic centimeter, at 1 mg. intervals. These were needed to prepare the colorimetric absorption curve and to use as checks in subsequent determinations of unknowns. Since sodium chloride was used as a standard, we have expressed our values as equivalents of sodium chloride rather than sodium. Other salts of sodium such as the carbonate, acetate, or citrate gave quantitative sodium equivalents.

The colorimetric determinations were made with a Fisher electrophotometer using the blue filter No. 425 and the 3 c.c. micro cells.

Preparation of Urine.—The urine should be refrigerated for several hours and then filtered before using. Salit reported that the bulk of the phosphates and urates can be precipitated by refrigeration. In our experience, if the urine was alkaline or was made alkaline by allowing ammonia to bubble through it, the phosphates were so completely precipitated by refrigeration that the addition of calcium hydroxide was not necessary. If albumin was present it was removed by addition of mercuric chloride or trichloracetic acid.

Preparation of Blood or Serum.—An equal volume of 20 per cent trichloroacetic acid should be added slowly and stirred vigorously to break up the clumps. This should be centrifuged and then the clear supernatant fluid used.

METHOD

Pipette 1 c.c. clarified urine, deproteinated serum, or sodium-containing solution into a 15 c.c. centrifuge tube.

Add 4 c.c. uranyl zinc reagent, freshly filtered.

Add 2 c.c. 95 per cent alcohol. (Use volumetric pipettes for these three solutions.)

Stir or invert the tubes to obtain thorough mixing and then allow to stand for twenty to thirty minutes.

Centrifuge. Decant the clear supernatant into the colorimeter absorption cell. If there is a pellicle of floating crystals, it may be eliminated by decanting the first few drops and then pouring the rest of the solution into the absorption cell.

Determine the color intensity with the photometer.

When urine is used as the unknown, there must be a correction for the yellow color of the urochrome. This is done by making up a blank tube with 1 c.c. of the urine specimen, 4 c.c. of water, and 2 c.c. of alcohol. The photometer reading of this blank is subtracted from the reading of the uranyl solution containing the urine.

The sodium content of the unknown is determined by interpolating the corrected photometer reading on the absorption curve prepared from the standard sodium solutions. The type of curve obtained is illustrated in Fig. 1. The scale values will be different for each photometer and for the absorption cells unless they are perfectly matched.

One or two standard solutions and a blank have been included with each determination to be certain that the conditions of the test and the solutions have not changed. The greatest variations observed in multiple determinations on standard solutions are indicated by the bars above and below the curve in Fig. 1. An example of the colorimeter readings and the sodium values for two urine specimens is given in Table I.

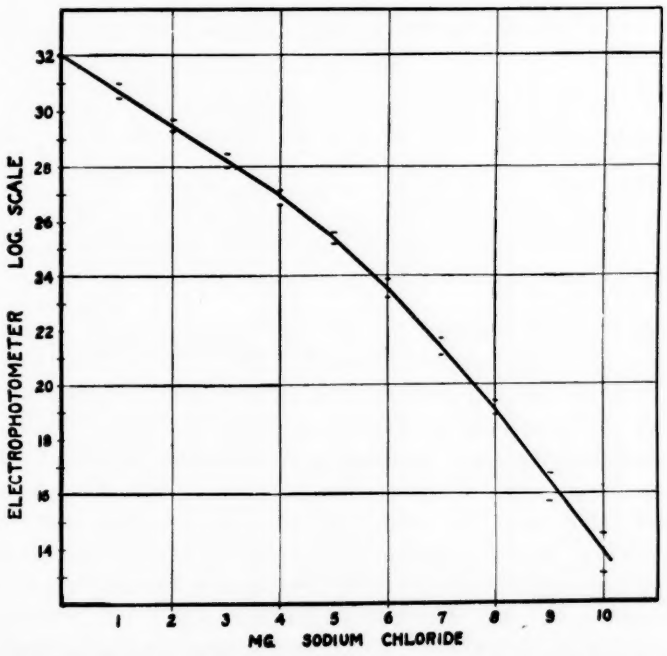

Fig. 1.—Absorption curve of uranyl zinc acetate reagent after precipitation by increasing quantities of sodium chloride. Obtained with a Fisher electrophotometer using blue filter No. 425 and 3 c.c. micro cells.

This colorimetric method was checked gravimetrically by mixing the solutions in a Jena filter and washing and drying the triple salt and then determining its weight. The method was that of Butler and Tuthill except for the proportion of the solutions and the addition of alcohol. The results of one trial using blood serum and standard solutions of sodium chloride are shown in Table II. In general, there was close agreement of values for each solution.

When sodium chloride values are less than 1.5 mg. per cubic centimeter, the difference between colorimeter readings is only 0.6 to 0.8 for 0.5 mg. differences in concentration. The absorption cells that were supplied with the electrophotometer varied as much as 0.6, so it was necessary to use the same cell for each color reading to detect small differences. To insure that the cell was in the

TABLE I. EXAMPLE OF PHOTOMETRIC READINGS OBTAINED ON URINE SAMPLES AND STANDARD SOLUTIONS OF SODIUM CHLORIDE AFTER TREATMENT WITH URANYL REAGENT AND CALCULATED SODIUM CHLORIDE CONCENTRATION IN URINES

	PHOTOMETER READING	COLOR CORRECTION	CORRECTED READING	CALCULATED NACL (MG./C.C.)
Urine No. 1	28.8	2.8	26.0	4.6
Urine No. 2	24.4	6.3	18.1	8.4
Blank	32.6	Observations made to be certain that conditions of the test and the solutions have not changed		
Standard NaCl				
5 mg./c.c.	25.3			
10 mg./c.c.	14.6			

TABLE II. COMPARISON OF GRAVIMETRIC AND PHOTOMETRIC ESTIMATIONS OF SODIUM CHLORIDE IN BLOOD SERUM AND IN STANDARD SOLUTIONS

SOLUTIONS TESTED	OBSERVATIONS		CALCULATED NaCl (MG./C.C.)	
	GRAVIMETRIC MG.	PHOTOMETRIC READING	GRAVIMETRIC	PHOTOMETRIC
Serum	114.0	26.6	8.50	8.40
Standard solutions (mg./c.c.)				
10.0	267.0	13.7	9.97	10.05
5.0	138.0	24.8	5.10	5.30
1.5	40.2	30.3	1.50	1.50
1.0	27.5	31.1	1.02	0.90
0.5	13.6	31.7	0.50	0.45

same position for each reading, a point was heated in a Bunsen flame, and a small bleb was blown in the side of the cell. This bleb fitted into a notch that had been cut in the carrier and assured exact orientation. By rinsing the cell with small portions of the solution to be read, any significant dilution by the remains of the previous solution was avoided and duplicate samples usually checked within 0.2 on the photometer scale. The curve is steeper when sodium chloride values exceed 4 mg. per cubic centimeter and differences in readings are more significant. Since, in our experience with urines, the sodium chloride concentration never exceeded 10 mg. per cubic centimeter, the curve was not extended beyond that point. Casual observations indicate that it can be used at least up to 15 mg. per cubic centimeter. It cannot be used to the theoretical zero because the yellow triple salt is soluble when the concentration of the reagent is reduced beyond a critical level.

SUMMARY

A simplification of the uranyl zinc method for determining sodium is described.

The method has been found suitable for the routine testing of urine and blood.

The method can be used when sodium chloride concentrations are as low as 0.5 mg. per cubic centimeter. Estimations by this colorimetric method have usually checked within 2 per cent of those obtained by the gravimetric method.

The author is indebted to Miss Donna MacLachlan and Miss Amanda Piper, for technical assistance in testing this method.

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A DIRECT PHOTOELECTRIC METHOD FOR THE DETERMINATION OF SERUM CALCIUM

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ONE of the disadvantages of the titrimetric methods for the determination of serum calcium with potassium permanganate is the difficulty encountered in reading the end point when the usual visual procedures are used. It should be possible to avoid this disadvantage by the application of colorimetric or spectrophotometric techniques to the analysis of serum calcium and at the same time to effect a considerable saving of time. Colorimetric methods for calcium have been based on the final precipitation of the element, as the alizarinate,[1] phosphate,[2-4] tungstate,[5] or oxalate.[6] More recently a method has been developed for the determination of calcium by precipitation as the oxalate and the spectrophotometric measurement of the permanganate.[7]

Some of the afore-mentioned methods, as colorimetric methods, have been used to a considerable extent for the special purpose for which they were developed. Their general limitations are not well established, and their applicability to the determination of serum calcium has been studied in only a few instances.

Sendroy[6] developed a photoelectric method for the determination of serum calcium based on the reduction of ceric sulfate and the measurement of the yellow color after the addition of excess potassium iodide. This method appears to give accurate results in the hands of an experienced analyst, however, it is rather laborious for the routine analysis of large numbers of samples, and it employs indirect equivalent relations and calculations.

The application of the permanganate method to the photoelectric determination of serum calcium appeared to offer several advantages over existing methods for serum calcium. The method presented in this paper is based on the precipitation of calcium as the oxalate and the photoelectric or spectrophotometric measurement of the amount of potassium permanganate reduced by using an excess of this reagent. By this method serum calcium may be determined with a high degree of accuracy on a relatively large number of samples with any one of a number of modern photoelectric colorimeters or spectrophotometers.

Reagents.—

Sodium oxalate solution. (Prepared by dissolving approximately 6 Gm. of sodium oxalate in 1 liter of distilled water.)

2 per cent ammonium hydroxide.

Approximately 5 N sulfuric acid.

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Standard potassium permanganate solution—approximately 0.01 N. (This may be used without standardizing provided a calibration curve is prepared with each new solution. The stability of the potassium permanganate can be conveniently checked at intervals with standard calcium oxalate. It may, however, be more convenient to prepare one standard curve for a specific normality and thereafter to adjust each new solution to that particular normality.)

Procedure.—To a 15 c.c. graduated conical centrifuge tube add 2 c.c. of serum that is free from erythrocytes plus 1 c.c. of the solution of sodium oxalate. Mix and allow to stand overnight to insure complete precipitation of the calcium oxalate. Centrifuge for five minutes at about 2,600 revolutions per minute. This packs the precipitate of calcium oxalate firmly enough to permit siphoning off all but about 0.25 c.c. of the serum and excess oxalate solution. This is conveniently done by using a capillary tube with an upturned tip. Resuspend the precipitate in the remaining solution and then add 5 c.c. of 2 per cent ammonium hydroxide from a pipette so that the sides of the tubes are rinsed to remove residual oxalate. Centrifuge at about 2,600 r.p.m. for five minutes. Repeat the washing procedure two more times or until all residual oxalate has been removed.

After washing, dissolve the precipitate in 5 c.c. of 5 N sulfuric acid. Place the centrifuge tubes containing the dissolved calcium oxalate in a boiling water bath for five or six minutes. Add exactly 2 c.c. of the potassium permanganate solution and dilute to 10 cubic centimeters. Let stand for five minutes, but no longer than fifteen minutes, before making spectrophotometric or colorimetric readings at 530 millimicrons.

Calibration Curve.—Calcium sulfate is used for preparing the calibration curve. A series of tubes is set up containing the following amounts of calcium: 0.10, 0.14, 0.18, 0.20, 0.22, 0.26, 0.30 and 0.34 milligram. The standard is then treated exactly as described for the serum. The density or transmittance, depending on which reading is taken, is plotted along the ordinate and the amounts of calcium along the abscissa of the graph. Calcium levels ranging from 5.0 to 17.0 mg. per 100 c.c. of serum can be read from the graph. Density measurements made with the Beckman spectrophotometer and transmittance measurements made with an Evelyn photoelectric colorimeter showed that Beer's law holds precisely over this range.

The accuracy of the procedure and the standard curve prepared with calcium sulfate may be verified with a standard calcium oxalate solution. The standard calcium oxalate is made up in 5 N sulfuric acid and added to the centrifuge tubes at calcium concentrations ranging from 0.1 to 0.34 milligram. Additional 5 N sulfuric acid is added to bring the volume in each tube up to 5 cubic centimeters. These are then treated as described for the serum following the solution of the oxalate. The spectrophotometric or colorimetric readings should check exactly with the readings for the calcium sulfate standards. The constancy of the potassium permanganate solution can be conveniently checked from time to time by the standard calcium oxalate.

Wave Length.—Transmittance minima for dilute permanganate solutions have been reported ranging from 526 to 530 millimicrons. Scott and Johnson[7] using a Coleman spectrophotometer reported that the transmittance minimum for dilute permanganate was 529 millimicrons. Using a Beckman quartz spectrophotometer, we have found that dilute permanganate solutions containing the same amount of acid as in the procedure for calcium have a maximum density at 529 to 530 millimicrons. With the Evelyn photoelectric colorimeter we have used a filter with transmittance maxima at 540 millimicrons.

TABLE I. RECOVERY OF CALCIUM ADDED TO SERUM

SAMPLE	VOLUME OF SERUM (C.C.)	CALCIUM CONTENT OF SAMPLE (γ)	CALCIUM ADDED (γ)	TOTAL (γ)	FOUND (γ)	FOUND (%)
1	2.0	252	80	332	334	100.6
2	2.0	213	80	293	301	102.8
3	2.0	226	80	306	301	98.4
4	2.0	238	100	338	352	104.1
6	2.0	250	80	330	328	99.4
6	1.6	222	64	286	268	93.7
6	1.2	164	48	212	200	94.4
7	2.0	234	83	317	332	104.7
8	2.0	242	83	325	343	105.5
8	1.6	221	66	287	281	97.9
9	2.0	257	83	340	358	105.5
9	1.6	220	66	286	271	94.7
10	1.2	179	48	227	235	103.5
11	2.0	262	80	342	345	100.9
11	1.2	176	48	224	235	104.9
12	2.0	240	80	320	331	103.4
12	1.6	218	64	282	276	97.9
13	2.0	201	80	281	283	100.7
14	2.0	240	80	320	319	99.7
14	1.6	195	64	259	252	97.3
14	1.2	156	48	204	209	102.4
15	2.0	229	80	309	309	100.0
15	1.6	195	64	259	277	106.9
15	1.2	154	48	202	209	103.5
16	2.0	256	80	336	340	101.2
16	1.6	241	64	305	301	98.7
16	1.2	194	48	242	245	101.2
	Average					100.9
	Standard deviation					3.6

Recovery Experiments.—Serum was obtained from various species by allowing the freshly drawn blood to clot, storing it in the refrigerator for a few hours. Each sample of serum was analyzed for calcium according to the technique described. A known amount of calcium, as calcium sulfate, was then added to the serum of each sample and analysis run to measure the recovery. These data showing the recovery of calcium added to serum are presented in Table I. The mean recovery of added calcium measured on the Beckman spectrophotometer was 100.9 per cent. The relatively small variation in the per cent recovery is apparent from the standard deviation of ±3.6. There is no evidence of any constant error in the method, as the per cent of calcium recovered for individual samples of serum was neither consistently above nor below the expected value.

Comparison of Values Obtained With Beckman and Evelyn Instruments.—Samples of serum from eighteen different animals were analyzed for calcium. Measurements were made on a Beckman spectrophotometer and on an Evelyn photoelectric colorimeter. Expressed on the basis of milligrams of calcium per 100 c.c. of serum, the mean value as determined with the Beckman spectrophotometer was 11.7 and with the Evelyn photoelectric colorimeter, 11.8.

Comparison of Values Obtained Spectrophotometrically and by Electrometric Titration.—The values for serum calcium by the spectrophotometric procedure were compared with the values obtained by electrometric titration of the oxalic acid with 0.01 N potassium permanganate using a Fisher titrimeter. Each sample of serum was set up in quadruplicate and treated according to the procedure outlined, except that the oxalic acid of two of the tubes was measured by electrometric titration with standard permanganate solution. The values for serum calcium as measured spectrophotometrically and by electrometric titration are recorded in Table II. The values obtained by the two procedures agree very well for each sample, the maximum variation being 0.4 mg. of calcium per 100 c.c. of serum. While only the averages for the duplicates are presented in Table II, it is significant that the magnitude of the variation between duplicate determinations was of the same order as the variation between the two procedures.

TABLE II. COMPARISON OF SERUM CALCIUM VALUES DETERMINED SPECTROPHOTOMETRICALLY AND BY ELECTROMETRIC TITRATION

SAMPLE	SPECTROPHOTOMETRIC (MG. PER 100 C.C.)	ELECTROMETRIC TITRATION (MG. PER 100 C.C.)
1	11.0	10.9
2	11.4	11.2
3	12.1	12.0
4	11.1	11.2
5	12.7	12.7
6	14.6	14.4
7	13.0	13.4
8	11.6	11.4
9	11.5	11.3
10	11.2	11.1
11	11.4	11.8
12	10.7	11.0
13	12.0	12.0
14	11.0	10.9
15	12.5	12.2
Average	11.8	11.8

SUMMARY

A method is described for the spectrophotometric determination of serum calcium. The calcium is precipitated as the oxalate and measured by the extent of the reduction of a constant amount of potassium permanganate by means of a spectrophotometer or photoelectric colorimeter. The values for serum calcium by this procedure agreed very well with the values obtained by electrometric titration with standard permanganate solution.

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A RAPID BEDSIDE TEST FOR THE DETECTION OF HYPOGLYCEMIA

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IN CERTAIN cases the diagnosis of hypoglycemic reaction can be quickly made from a suggestive history and consistent physical findings. Unfortunately, not all cases present such a simple problem; the history is often unobtainable or misleading, and the physical signs are inconclusive. A quantitative determination of the blood glucose concentration usually requires (in an average, reasonably well-equipped hospital) twenty minutes or more. (An analysis can, of course, be made more quickly if other analyses are already in progress.)

Because of deleterious effects, especially upon the central nervous system, it is imperative that the question of hypoglycemia in a patient with suggestive findings be settled rapidly. This fact has given rise to the practice of administering glucose intravenously as a therapeutic test. In an appreciable number of cases, this is a rational and useful method of making the diagnosis and initiating therapy.

But the need for an alternative procedure is apparent from the following considerations. The number of patients admitted in unexplained coma or with other findings suggestive of hypoglycemia (deserving, therefore, immediate investigation) is large in proportion to the number who are found actually to have hypoglycemia. A therapeutic test requires a heavy dose of intravenous glucose, since at times a surprisingly large amount must be given rapidly to produce an effect in a patient with hypoglycemia. A patient admitted to the Rhode Island Hospital required 70 c.c. of 50 per cent glucose solution intravenously before showing any response. The only practical way of giving the needed amount is to use a very concentrated solution. One cannot draw conclusions from failure to obtain a response to a small amount of the concentrated solution. Since large amounts of concentrated, hypertonic glucose solutions are definitely dehydrating, hypoglycemia should be investigated by some other method in the case of poorly hydrated patients with suggestive findings, not an uncommon situation both in patients with hypoglycemia (for example, Addisonian) and normoglycemia.

The method described here utilizes commercial powder (containing oxidized bismuth and alkali) employed to detect glucose in urine. Such powder turns gray or black when exposed to a solution containing a sufficient amount of reducing agent. Whole blood is used; the proteins are precipitated by copper. Since much of the time consumed in blood glucose determinations is taken up by the gravity filtration of the viscous mixture, a simple method of pressure filtration is substituted here.

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A short test tube (tubes approximately 7 by 1.5 cm. are satisfactory), containing 200 mg. of dry $CuSO_4 \cdot 5H_2O$, is marked to contain 5 c.c. of whole blood. Blood taken by venipuncture is introduced directly into the tube to the 5 c.c. mark, and sufficient 3 N NaOH is added from a dropper or pipette to make the mixture just alkaline to litmus; 0.4 c.c. (or 6 drops from the average dropper) is required. It has been found that the reaction can be carried out most rapidly by using a dropper which has already been calibrated in previous tests, with the aid of litmus. Once the required number of drops from a given dropper is known, it can be used without the necessity of checking with litmus, and alkalinization can be performed more rapidly and conveniently than with a graduated pipette.

The mixture is stirred with a small wooden reamer or thin glass rod, the tube corked, and mixing completed by shaking. Part of the mixture is then poured into the barrel of a 10 c.c. syringe, at the bottom of which two or three disks of filter paper have been placed. The disks must fit the internal diameter of the syringe closely. (Disks used in penicillin assay have been found satisfactory.) The plunger is then inserted and pushed down the barrel, compressing the mixture, so that a clear filtrate rapidly escapes through the nozzle. The first 2 or 3 drops of filtrate are discarded, and the next drop is allowed to fall on a *large excess* of Galatest powder. If within a period of forty-five seconds, a definite gray or black color appears, the reaction is considered normal. Failure of a definite gray or black to appear constitutes an abnormal (hypoglycemic) result. Since any filtrate may discolor the powder brown or yellow, an unequivocal (but not necessarily intense) black or gray must develop, if the reaction is to be considered normal. However, if the test is performed against a white background, this distinction is usually very easy to make, even if only a faint gray color develops.

In developing this test, whole oxalated blood was incubated for twenty-four hours at 37° Centigrade. Various amounts of glucose were then added to portions of the autoglycolysed blood. The artificially set blood glucose concentrations were checked by the method of Benedict.*† At the Rhode Island Hospital this method has given a normal range of 50 to 100 mg. per 100 cubic centimeters. For all practical purposes, a value less than 50 mg. per cent, or, at any rate, less than 45 mg. per cent, may be considered abnormal. In a series of bloods, with values set, as previously described, from 8 to 67 mg. per cent, the rapid method outlined herein gave abnormal reactions with all bloods containing 44 mg. per cent or less and normal reactions with all bloods containing 53 mg. per cent or more. Repetition of the tests substantiated this.

The speed with which the blood glucose decreases is a factor in determining how low the concentration may fall before symptoms supervene; therefore, an occasional patient whose low value is not actually the cause of his symptoms may be diagnosed as abnormal by this test. In such a case, however, the danger

*Benedict, S. R.: The Analysis of Whole Blood. II. The Determination of Sugar and of Saccharoids (Nonfermentable Carbohydrate Reducing Substances), J. Biol. Chem. 92: 19-41, 1931.

†Methods which determine blood "glucose" actually determine glucose plus a certain percentage of nonglucose reducing substances. The latter represent only a few milligrams per cent in the Benedict method.

point would be close, so the diagnosis and resultant therapy could not be considered seriously in error. A value extremely close to 50 mg. per cent is difficult to read; a patient whose blood yields such a value may receive needless glucose, but such cases should be rare. The test as described here will detect all cases with blood glucose sufficiently low to cause serious effects, while ruling out the vast majority of patients with normal or elevated values.

The apparatus needed, prepared in advance, can conveniently be kept in a small container on the ward and the test carried out at the bedside. Under these circumstances, performance requires three to three and one-half minutes from the time blood is drawn from a vein.

The Galatest brand of urine-testing powder was used because it was readily available at the time of these experiments. Nothing written in this article should be construed as a specific recommendation for this particular brand. It cannot be stated now whether any other powder of similar composition would be superior or inferior.

In conclusion, it should be stressed that this test is useful solely to discover quickly whether hypoglycemia is present in a patient with suggestive findings for whom the needless intravenous administration of a large amount of glucose would be harmful. It is intended to supplement, but certainly not to replace, quantitative blood glucose determinations, which are indispensable in the proper study of hypoglycemia and its course.

SUMMARY

1. A method whereby the diagnosis of hypoglycemia can be made or ruled out in three to three and one-half minutes is described.

2. Mention is made of occasions where this method is preferable to the use of a therapeutic test with intravenous glucose solution.

AN ABBREVIATED SPECTROPHOTOMETRIC TECHNIQUE FOR DETERMINING THE OPTIMAL CONCENTRATION OF AMBOCEPTOR

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WITH THE TECHNICAL ASSISTANCE OF REBECCA GOODMAN AND HELEN CONWAY

THE optimal concentration of amboceptor has been defined as that beyond which further increase fails to enhance appreciably the hemolytic activity of complement.[1a] The demonstration of this optimum depends largely upon the accuracy with which changes in the activity of complement are measured and accordingly follows the application of a quantitative method in the titration of complement for fixation studies.[2] The method in question employs the end point of 50 per cent hemolysis.

Use of the 50 per cent end point demands accuracy in determining the percentage of hemolysis in titrations. The required degree of precision has been obtained through the use of prepared color standards.[1b] More recently, adaptations of the spectrophotometer have contributed to the accuracy and facility of the determination,[3, 4] and the employment of this instrument in conjunction with a simple graphic method has permitted the quantitative determination of the 50 per cent unit of complement without constructing the curve of hemolysis.[4] Continued studies at this laboratory have included the application of similar methods to the standardization of amboceptor. As a result, there has been developed a simplified technique for determining the optimal concentration of the amboceptor. The procedure employs the spectrophotometer in measuring percentage of hemolysis, and a standard curve is applied in determining the 50 per cent unit of complement when varying concentrations of amboceptor are used. Since the method has yielded accurate and reproducible results in our hands, a description may be of some interest to those engaged in complement fixation studies.

MATERIALS AND METHODS

Spectrophotometer.—The Coleman Junior Clinical Spectrophotometer Model 6* is used. All measurements are made in terms of optical density at 580 millimicrons. The use of a 12 mm. adapter permits the employment of selected 12 by 75 mm. serologic tubes as cuvettes. Tubes of uniform internal diameter are selected on the basis of snug fit with a metal rod cut to 10 mm. diameter.

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*Manufactured by the Coleman Electric Company, Maywood, Ill.

A total volume of 2.0 c.c. in these tubes is required to bring the level of the contents well above the slit through which light passes before striking the photoelectric cell. This volume represents twice that used in diagnostic tests at this laboratory and occasions the use of double volumes (2V) of complement, salt solution, amboceptor, and sheep's cells in spectrophotometric determinations. The result of a titration carried out under the latter conditions is transferable without correction to the conditions of the diagnostic tests employing unit volumes (V) of the reagents since, as in the amboceptor titration to be described, it is expressed directly in terms of the required concentration, or dilution, of the reagent titrated.

Diluent.—Salt solution, 0.85 per cent, buffered to pH 7.3 with 0.005 M phosphate is employed as diluent for all reagents. In practice, a stock solution containing 170.0 Gm. of NaCl, 2.7 Gm. of KH_2PO_4, and 11.3 Gm. of Na_2HPO_4 to the liter is diluted 1:20 in distilled water before use.

Sheep's Red Blood Cells.—A 2 per cent suspension is prepared from blood preserved by aseptic collection into an equal volume of modified Alsever's solution.[5] The required volume of blood is washed three times with salt solution in 15 c.c. centrifuge tubes. An International centrifuge with head No. 240 is used. After a final centrifugation for ten minutes at 2,000 r.p.m., the volume of packed cells is read, and the 2 per cent suspension is prepared by diluting to 50 volumes with salt solution. It is then standardized spectrophotometrically. The suspension is mixed thoroughly by rotation, after which a 0.4 c.c. sample is measured accurately into a 12 by 75 mm. tube and lysed by the addition of 1.6 c.c. of distilled water. The tube is spun for five minutes at 2,000 r.p.m., and the optical density is determined at 580 mμ against a tube of water as a blank. Suspensions yielding an optical density of 0.500 ± 0.01 are accepted. Corrections to this reading are made by adding or removing salt solution, the required final volume of the suspension being determined according to the relation

$$V_2 = \frac{V_1 OD_1}{0.500}$$

where V_1 is the volume of the suspension yielding the observed optical density OD_1, and V_2 is the final volume required to yield an optical density of 0.500. Salt solution is removed, when necessary, by spinning a suitable amount of the suspension, removing the required volume of supernatant salt solution, and resuspending the remaining supernate and cells in the parent 2 per cent suspension.

In the Coleman Junior Spectrophotometer, using the standardized 12 by 75 mm. tubes, a mean optical density of 0.500 at 580 mμ has been found to correspond with a cell concentration of 500,000 per cubic millimeter. If other instruments or cuvettes are employed, the mean optical density equivalent to this cell count should be redetermined.

Amboceptor.—Amboceptor produced by the immunization of rabbits is preserved by lyophilization or by the addition of an equal volume of neutral glycerol, chemically pure. A stock solution representing a 1:100 dilution of original serum is prepared by adding 99.0 c.c. of salt solution to 1.0 c.c. of

rehydrated serum, or 98.0 c.c. of salt solution to 2.0 c.c. of glycerolized serum. It may be stored safely at 3 to 6° C. for one week.

Complement.—Commercial, lyophilized guinea pig serum is used. The dried equivalent of 7.0 c.c. is rehydrated by the addition of 5.0 c.c. of the accompanying diluent which contains 2 per cent boric acid and 6 per cent sodium acetate. A stock 1:20 dilution sufficient for the day's tests is prepared by adding 19 volumes of salt solution to 1 volume of the rehydrated guinea pig serum.

Fresh, pooled guinea pig serum may be used with equally satisfactory results.

DETERMINATION OF THE OPTIMAL CONCENTRATION OF AMBOCEPTOR

Preparation of the Amboceptor Dilutions.—A 1:500 dilution of amboceptor is prepared by adding 8.0 c.c. of salt solution to 2.0 c.c. of the stock 1:100 dilution. According to our practice, an amboceptor showing an optimum at a dilution less than 1:500 is not considered of satisfactory titer. Varying quantities of 1:500 amboceptor and salt solution are combined as indicated in Table I to provide dilutions representing a suitable progression from 1:500 to 1:4,000. These dilutions are used to sensitize equal volumes of the cell suspension which have been measured accurately into separate tubes. It has been our custom to measure the 5.0 c.c. quantity of 1:500 amboceptor and corresponding volume of cells into separate 15 by 150 mm. tubes and the 1.6 c.c. quantities of the higher amboceptor dilutions and corresponding volumes of cells into separate 13 by 100 mm. tubes. The amboceptor dilutions are then poured into the respective tubes of cells; the two are mixed thoroughly by ten successive pourings, and at least ten minutes at room temperature are allowed for sensitization.[6]

TABLE I. PREPARATION OF AMBOCEPTOR DILUTIONS

	AMBOCEPTOR DILUTIONS						
	1:500	1:666	1:1000	1:1333	1:2000	1:2666	1:4000
Amboceptor 1:500 (c.c.)	5.0	1.2	0.8	0.6	0.4	0.3	0.2
Salt solution (c.c.)	0	0.4	0.8	1.0	1.2	1.3	1.4

Preparation of 1 Unit Complement.—Complement is titrated to the end point of 50 per cent hemolysis.[4] A 1:100 dilution is prepared by adding 4.0 c.c. of salt solution to 1.0 c.c. of the stock 1:20 dilution of complement. It is titrated as indicated in Table II, using the cells that have been sensitized with 1:500 amboceptor. Carefully measured quantities of the reagents are added in the order given, and the contents of each tube are thoroughly mixed. After incubation for thirty minutes in the water bath at 37° C., all tubes of the titration are spun for ten minutes at 2,000 r.p.m., and the optical densities of their supernates are determined spectrophotometrically at 580 millimicrons. The galvanometer of the instrument is set at "0" optical density (100 per cent transmission) with Tube 10 in the adapter, and the optical densities of Tubes 1 to 9 are determined successively. Tubes 7 to 9 contain an excess of complement calculated to yield 100 per cent hemolysis. The mean of their three optical densities is taken as the standard of reference. The percentage of hemolysis in each of the first six

tubes is 100 times the quotient obtained by dividing its optical density by the mean value for 100 per cent hemolysis. The optical densities and corresponding percentages of hemolysis in Table II represent a typical result.

TABLE II. COMPLEMENT TITRATION

	TUBE									
	1	2	3	4	5	6	7	8	9	10
Stock 1:20 complement (c.c.)							0.40	0.40	0.40	
1:100 complement (c.c.)	0.30	0.35	0.40	0.45	0.50	0.55				
Salt solution (c.c.)	0.90	0.85	0.80	0.75	0.70	0.65	0.80	0.80	0.80	1.20
Cells sensitized with 1:500 amboceptor (c.c.)	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80	0.80
Optical density *(a)*	0.196	0.282	0.370	0.439	0.456	0.490	0.502	0.500	0.495	
Mean optical density for 100 per cent hemolysis *(b)*								0.499		
Per cent hemolysis ($c = \frac{100a}{b}$)*	39.0	56.5	74.0	88.0	91.5	98.0				

*Calculated to the nearest 0.5 per cent.

The volume of 1:100 complement required for 50 per cent hemolysis is then determined by a precise graphic method which avoids construction of the hemolytic curve.[4] Other methods employing the construction of this curve may be used with excellent results.[1c, 3] Given x, the volume of 1:100 complement required for 50 per cent hemolysis, y, the dilution of stock 1:20 complement containing 1 unit in 0.4 c.c. is determined according to the relations

$$(1) \qquad \frac{y'}{100} = \frac{0.4}{x} \qquad \text{and} \quad (2)\ y = \frac{y'}{20}$$

where y′ is the dilution of undiluted complement containing one unit per 0.4 cubic centimeter. Substituting in Equation 2 the value of y′ derived from Equation 1, and simplifying, gives

$$(3) \qquad y = \frac{2}{x}$$

If, as in the titration of Table II, 0.332 c.c.* of 1:100 complement is the unit,

$$y = \frac{2}{0.332} = 6.024$$

and 1 unit complement for use in the amboceptor titration would be prepared by adding 5.02 c.c. of salt solution to 1.0 c.c. of stock 1:20 complement.

Technique of the Amboceptor Titration.—The amboceptor titration is carried out as illustrated in Table III. The reagents are added accurately in the

*The graphic method[4] in this instance gives 0.166 c.c. of 1:100 complement as the unit for the conditions of the diagnostic tests which employ unit volumes (V) of reagents. Since the amboceptor titration employs the double volumes (2V) required for spectrophotometric determinations, twice 0.166, or 0.332 c.c. of 1:100 complement, represent the unit under the latter conditions (see Spectrophotometer).

order given, and the contents of each tube are thoroughly mixed. Thirty minutes in the water bath at 37° C. are allowed for hemolysis, after which the tubes are spun at 2,000 r.p.m. for ten minutes. The optical densities of their supernates are determined spectrophotometrically. Tube 10 of the completed complement titration (Table II) is re-used to set the instrument at "0" optical density, and the predetermined mean optical density of Tubes 7 to 9 (Table II) serves again as the standard of reference for 100 per cent hemolysis. The percentages of hemolysis in Tubes 1 to 7 are calculated as for the complement titration. The results of a sample determination are given in Table III.

TABLE III. AMBOCEPTOR TITRATION

	TUBE						
	1	2	3	4	5	6	7
	DILUTION OF AMBOCEPTOR						
	1:500	1:666	1:1000	1:1333	1:2000	1:2666	1:4000
1 unit complement (c.c.)	0.40	0.40	0.40	0.40	0.40	0.40	0.40
Salt solution (c.c.)	0.80	0.80	0.80	0.80	0.80	0.80	0.80
Sensitized cells (c.c.)	0.80	0.80	0.80	0.80	0.80	0.80	0.80
Optical density *(a)*	0.274	0.259	0.251	0.211	0.146	0.075	0.026
Per cent hemolysis $(c = \frac{100a}{b})$*	55	52	50	42	29	15	5
Units of complement required for 50 per cent hemolysis (from Fig. 1, *A*)	0.97	0.99	1.00	1.05	1.15	1.30	1.56

*Calculated to the nearest 0.5 per cent.

If the 1 unit complement used in this titration has been correctly prepared, 0.4 c.c. should yield 50 per cent hemolysis of the cells sensitized with 1:500 amboceptor. Deviations from this percentage, while they do not invalidate the results of the amboceptor titration, should not exceed the range of 45 to 55 per cent hemolysis.

Determination of the Optimal Concentration of Amboceptor.—The number of complement units that would be required for 50 per cent hemolysis with the different dilutions of amboceptor is then determined from the percentages of hemolysis obtained with one 50 per cent unit in the titration. This determination is made by reference to the curve of Fig. 1, *A*, the construction of which is described in the next paragraph. Proceeding with Tube 1 of the titration and referring to Fig. 1, *A*, it is observed that if 55 per cent hemolysis is obtained with 1 unit of complement, 0.97 unit would be required for 50 per cent hemolysis. Values obtained in this way for Tubes 1 to 7 are included in Table III. They are plotted on cross-section paper, against the corresponding concentrations of amboceptor, and a smooth curve is fitted to the coordinate points. The method, as applied to the results of Table III, is illustrated in Fig. 1, *B*. It will be noted that coordinate points representing concentrations of amboceptor from 0.0004 to 0.0008 c.c. per 0.4 c.c. of the diluted reagent fall, for all practical purposes, on a straight line, while points representing lower concentrations deviate from

linearity. It has been our practice to select as optimal the lowest concentration of amboceptor falling on the apparently linear portion of the curve. In the present instance, such an optimum would be represented by a concentration of 0.0004 c.c. per 0.4 c.c. which corresponds with the 1:1,000 dilution. The dilution so selected should not cause agglutination of 2 per cent sheep's cells under the conditions in which these reagents are used in complement fixation tests. The optimal dilution need be redetermined only under conditions suggesting a deterioration of the amboceptor.

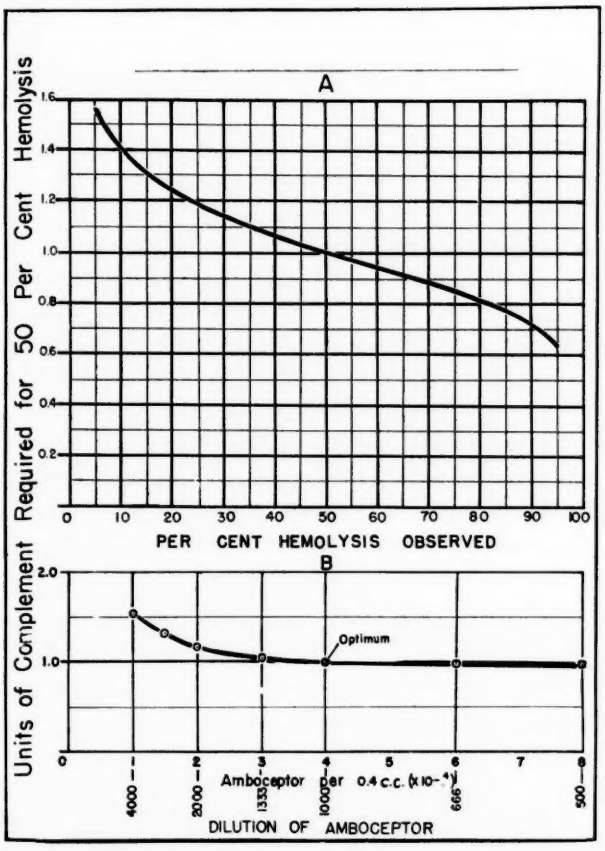

Fig. 1.

Construction of the Curve of Fig. 1, A.—The construction of this curve is based upon the observation that the curve of hemolysis is constant under given experimental conditions and may be expressed by the von Krogh alternation formula.[7] The application of this formula to the standard conditions of the complement titration at this laboratory has been described, and volume factors have been derived which define the 50 per cent unit of complement in terms of the quantities of the reagent yielding other degrees of partial hemolysis.[4] These factors are given in Table IV; plotted on arithmetic coordinates against the corresponding percentages of hemolysis, they yield the curve of Fig. 1, *A*.

TABLE IV. VOLUME FACTORS FOR CONSTRUCTION OF CURVE OF FIG. 1, *A*

PER CENT HEMOLYSIS OBSERVED WITH ONE UNIT OF COMPLEMENT	UNITS OF COMPLEMENT REQUIRED FOR 50 PER CENT HEMOLYSIS	PER CENT HEMOLYSIS OBSERVED WITH ONE UNIT OF COMPLEMENT	UNITS OF COMPLEMENT REQUIRED FOR 50 PER CENT HEMOLYSIS
5	1.56	55	0.97
10	1.40	60	0.94
15	1.30	65	0.91
20	1.24	70	0.88
25	1.18	75	0.85
30	1.14	80	0.81
35	1.10	85	0.77
40	1.06	90	0.72
45	1.03	95	0.64

During the course of these studies, it was observed that as the concentration of amboceptor is reduced below the optimum there occurs a slow increase in slope of the hemolytic curve as expressed by the constant 1/n in von Krogh's formulation. While derivation of the volume factors corresponding to these alterations in the value of 1/n would make possible slightly more accurate estimations of the 50 per cent unit of complement in the range of amboceptor deficiency, the observed error in the immediate range of the optimum is so slight as not to interfere with the selection of an optimal concentration of amboceptor by the present method. Moreover, the practical use of factors appropriate to varying degrees of amboceptor deficiency would presume knowledge of the optimum, which is itself the object of the determination, and they could accordingly not be applied *ab initio* in the standardization of an unknown antiserum.

SUMMARY AND CONCLUSIONS

A simplified technique has been developed for use in determining the optimal concentration of amboceptor for use in complement fixation tests. The method obviates both the preparation of color standards for measuring percentage of hemolysis and the construction of the hemolytic curve in determining the 50 per cent unit of complement. The percentage of hemolysis observed with one 50 per cent unit of complement in the presence of different concentrations of amboceptor is determined spectrophotometrically. The number of complement units required for 50 per cent hemolysis in each instance is estimated simply by reference to a standard curve which is represented graphically. The abbreviated method has been found to yield accurate and reproducible results.

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BOOK REVIEWS

Experimental Hypertension. Vol. 3. Edited by *Roy Waldo Miner*. Results of a conference on this subject held by the Section of Biology of the New York Academy of Sciences, Feb. 9 and 10, 1945, New York City. Special publication of the New York Academy of Sciences, New York, 1946. Price, $3.75. Cloth, with 180 pages.

This publication consists of nine major papers presented at a conference on experimental hypertension by nineteen participants and covering as many viewpoints on the problem. In the Preface, Irvine H. Page states: "Investigation in the medical sciences often passes through several strata of development: the first, awareness that a problem exists, but widespread indifference to it; the second, attempted formulation of the problem along with much speculation; the third, widespread intellectual and emotional disagreement among those concerned with putting the problem to experimental test; fourth and last, agreement about the fundamental nature of the mechanism, application of the new knowledge to the care of patients, and commercialization. The testimony of the conference will leave little doubt that the third phase is beginning. It is a healthy state and one rich with promise."

The "intellectual and emotional disagreement" is summarized at the end of the book by William Goldring. There was a division of opinion on almost all points discussed at the conference. Such diversity of views does not make for easy reading, when presented in book form, in spite of denoting a "healthy state."

Although the conference was concerned with experimental hypertension, most of the papers considered various aspects of hypertension induced by interference with renal blood supply, with little discussion of hypertension induced by interference with various nervous pathways or by other means. Furthermore, it was naturally impossible to confine the discussion to experimental hypertension, per se, to the exclusion of arterial hypertension in man. However, most of the participants were careful to avoid applying the results of experimental work to human beings. Thus the book opens with a brilliant introductory lecture by Goldblatt in part of which the similarity of experimental renal hypertension and arterial hypertension in man is upheld. The book closes with a statement by Goldring, Chasis, and Smith, in which the opposite viewpoint is taken, that is, that the diminished renal blood flow which accompanies essential hypertension in man is merely a secondary effect "resulting from the action of a humoral pressor agent of unknown origin." Between these two positions the rest of the conference carefully avoided any commitment.

The various papers are well presented in a form suitable for publication in a journal. However, the wide divergence of views on a subject which invades so many fields gives the reader a sense of discontinuity. This is further amplified by the publication of full discussions of each paper.

As a reference book for workers in this specialized field, this publication presents the later ideas and summarizes the earlier work done by most of the groups who have contributed to our knowledge of experimental hypertension. However, it is doubtful whether this collection of papers would interest the casual reader, being just what it is meant to be—publication of a symposium on a highly specialized subject which is just beginning to open up and is therefore still confused.